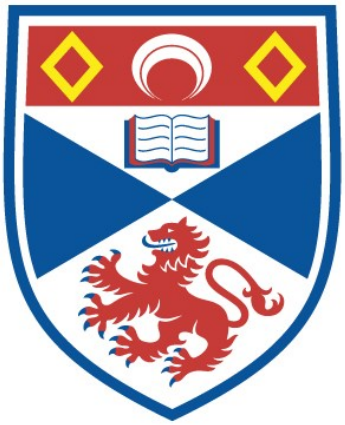
THE MEASUREMENT OF THYROID STIMULATING HORMONE IN BODY FLUIDS. HISTOMETRIC AND RADIOMETRIC TECHNIQUES USING THE XENOPUS LARVA

Judith Rosemary Brown

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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THE MEASUREMENT OF THYROID STIMULATING HORMONE IN BODY FLUIDS: HISTOMETRIC AND RADIOMETRIC TECHNIQUES USING THE XENOPUS LARVA

by

Judith R. Brown

Thesis presented for the Degree of Doctor of Philosophy in the Faculty of Science of the University of St. Andrews.

DECLARATION

I hereby declare that the work recorded in this Thesis has been carried out by me, except where otherwise stated, and that the Thesis is of my own composition. I further declare that it has not been submitted in any previous application for a higher degree.

RESEARCH CAREER

The research work recorded in this Thesis was carried out between the years 1954 and 1958, partly as a research student at the Gatty Marine Laboratory, University of St. Andrews and partly as a member of the scientific staff of the Medical Research Council at the Clinical Endocrinology Research Unit, University of Edinburgh.

SUPERVISOR'S CERTIFICATE

I certify that Judith Rosemary Brown has fulfilled the conditions laid down in the regulations of the Degree of Ph.D., under Ordinance No: 16 of the University Court of the University of St. Andrews and she is accordingly qualified to submit this Thesis for the Degree of Doctor of Philosophy.

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C O N T E N T S

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PUBLISHED PAPERS

Measurement of thyrotrophic hormone (TSH) using the *Xenopus* tadpole and ^{131}I.

The biosynthesis of thyroxine and 3:5:3' - Triiodothyronine in larval and adult toads.

... ...

PREFACE

There is no doubt that, of all the pituitary hormones, Thyroid Stimulating Hormone (TSH) is the most difficult to measure at the level of concentration at which it is normally present in body fluids; this is reflected in the inordinate number of methods of assay which are described in the literature. One of the most successful techniques, and one which has best stood the test of time, is that of D'Angelo (see below), using tadpoles of Rana pipiens. Unfortunately, R. pipiens is not available in this country and, even in the United States, its tadpoles can only be obtained seasonally. On the other hand, Xenopus laevis is well established as a laboratory animal in Britain and its tadpoles can be obtained at all times of the year.

The work described in this thesis was undertaken in an attempt to apply methods similar to those of D'Angelo, using the Xenopus tadpole. These methods are dependent on the measurement of histological stimulation of the larval thyroids by TSH. The investigations have been extended to include new techniques in which physiological stimulation of the larval thyroids by treatment with TSH is assessed by means of radiometric criteria.

It was intended that the methods should be suitable for routine application in clinical investigations. Accordingly, the findings have been assessed on the basis of the requirements for such an assay.

PART I

THE MEASUREMENT OF THYROID STIMULATING HORMONE (TSH):

A CRITICAL REVIEW OF THE LITERATURE

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THE MEASUREMENT OF THYROID STIMULATING HORMONE (TSH): A CRITICAL REVIEW OF THE LITERATURE. *

INTRODUCTION

During the past thirty years, more than seventy assay methods for TSH have been proposed. Many of these are reasonably satisfactory for estimations in pituitary extracts, but few are sufficiently sensitive to detect the very small quantities of the hormone present in blood and urine in normal and pathological conditions. The literature on this subject has previously been reviewed by Junkmann (1936), White (1944), (1946), Adams (1946), Lamberg (1955), Sonenberg (1958) and Loraine (1958). The purpose of the present article is to review the reliability criteria of these techniques, to discuss their practicability in clinical research and to indicate their possible value in the diagnosis prognosis and treatment of disease in man.

The reliability criteria discussed are precision, specificity and sensitivity, definitions of which will be found elsewhere (Borth, 1952, Loraine, 1958). A section is also included in which the importance of expressing results of TSH assays in International Units rather than "animal units" is emphasised.

Assay methods will be divided into 5 groups termed respectively:-

A. Gravimetric methods.

B. Histometric methods.

C. Metabolic methods.

D. Radiometric methods.

E. *In vitro* methods.

* The Review of the literature (pages 1 - 25) has been accepted for publication by Acta Endocrinologica, Copenhagen.

STANDARDISATION

One of the main difficulties in the field of TSH assay is the lack of standardisation. The original Junkmann-Schoeller unit, defined as "that amount of thyrotrophin extract required to produce definite signs of histological stimulation in one out of two guineapigs after three daily injections" (Junkmann & Schoeller, 1932), has been persistently used by a number of authors. Because the results of various investigations have been expressed in arbitrary "animal units" and because the potency quoted for these units has varied from laboratory to laboratory, it is not always possible to relate the sensitivity claimed for various methods to one another in terms of the International Standard. The wide variation in strain sensitivity in the test-animals, together with the use of only small numbers of animals, has been a further source of error in interpretation of results. This is illustrated by a comparison of the various "animal units" defined by different authors (Junkmann & Schoeller, 1932, Heyl & Lacquer, 1935, Rowlands & Parkes, 1934, Rawson & Salter, 1940).

A standard of reference for thyrotrophin was originally set up in 1938 by the Health Organisation of the League of Nations. The International Standard for Thyrotrophin was established in 1954 (Musset & Perry 1955), and one International Unit (I.U.) was defined as the activity present in 13.5 mg. of the standard preparation. This is regarded as equipotent with the USP unit, the latter being the activity of 20 mg. of the USP reference substance, established in 1952.

Asboe-Hansen, Iversen & Wichmann (1952) considered 40 Junkmann-Schoeller units (JSu) to be equivalent to 1.1 USP units, but it is now generally accepted that 1 USP unit $\simeq$ 10 - 12 JSu (Hays & Steelman, 1955,

Querido, 1957, Bakke & Lawrence, 1956). For the purpose of comparison of the methods, it is proposed, wherever possible, to express sensitivity in terms of International milliunits (imu) per millilitre of the test-substance assuming that 1 I.U. = 10 JSu from the relationship quoted above.

A. Gravimetric Methods

1. Guineapig.

A number of methods for estimating TSH by thyroid weight increase in the young guinea-pig have been described (Loeb & Bassett, 1929-30, Rowlands & Parkes, 1934, Aron, 1930[a], 1930[b], Reece & Turner, 1937). The suggestion that this type of test might have a clinical application originated with Aron, (1930). He observed signs of thyroid stimulation after injection of untreated urine and studied the effects of urine extracts from patients with hypo- and hyperthyroidism. Serum and cerebrospinal fluid were also investigated (Aron, Van Canlert & Stahl, 1931). Thyrotrophic activity was shown to be increased in hypothyroidism and decreased in hyperthyroidism. These findings were extended by Bodart & Fellinger (1936) and Offret & Offret (1945) who detected TSH in the sera of normal subjects and in three patients with exophthalmos and also by Smith & Moore (1933), who were unable to detect TSH activity in the urine of four patients with thyrotoxicosis.

The test was placed on a more quantitative basis by Rowlands & Parkes (1934) working with large groups of animals. The Rowlands-Parkes "Unit", defined as "the amount of thyrotrophin which causes 100% increase in thyroid weight in guineapigs after five daily injections", was subsequently used as a reference unit by several groups of workers and their method employed in a series of tests on serum from normally pregnant women (Emmens, 1940).

2. Chick.

An improvement in the sensitivity of gravimetric estimations was obtained by using the young chick as test-animal (Cope, 1938, Kabac & Laipin, 1938, Smelser, 1938, Bergman & Turner, 1939, Fraenkel & Conrat, 1940). Cope (1938) investigated the activity present in extracts from human pituitary tissue and in urine to which exogenous TSH had been added. Bates (1941) studied the problem of strain differences in sensitivity. He showed that, of two strains of chick studied, one required a dose of TSH four times as great as the other to produce an equivalent degree of stimulation.

3. Other species.

The Carneau pigeon was proposed as a possible test-animal by Riddle (1931) and used as an alternative to the chick by Bates (1941). Miller (1938) studied the effect of TSH injections over long periods in the sparrow, Passer domesticus. He suggested that this might prove to be a suitable test-animal for TSH assay since the birds showed no signs of becoming insensitive after prolonged periods of injection; this is in marked contrast to mammals which become refractory after a relatively short period of time.

Comment.

Recent findings, using methods of improved sensitivity indicate that TSH concentrations in serum from euthyroid subjects are of an extremely low order. It is thus improbable that the apparent response obtained to injections of serum was a specific reaction to administered TSH. Methods based on gravimetric criteria require a prolonged injection period before any effect becomes apparent. Because of lack of sensitivity and long response-time they can have no application to the estimation of

TSH in body fluids.

B. Histometric Methods

1. Guineapig.

By far the largest group of assay methods consists of those based on histological criteria. In the first instance these were qualitative tests (Junkmann & Schoeller, 1932, Heyl & Laoquer, 1935). The method of Heyl & Laoquer (1935) is dependent on identification of the response obtained according to an arbitrary scale of degree of histological stimulation. Despite the subjective element involved, a modification of this technique is still in routine use in some laboratories in standardisation of pituitary extracts. This form of qualitative test, when applied to estimations on serum, yielded equivocal results (Del Castillo & Magdalena, 1931, McGinty & McCulloch, 1936). When serum from euthyroid subjects was studied, one out of four samples gave a positive response; some of the control animals, injected with saline, also showed signs of stimulation. Emmerson & Cutting, (1938) extracted urine by alcohol and acetone precipitation. Urinary excretion of TSH was shown to be higher in patients suffering from myxoedema of recent origin than in cases in whom the condition had been established for some time.

Increased mitotic activity was used as an index of thyroid stimulation by Bastenie & Zylbersac (1937). By this method measurable amounts of TSH were demonstrated in the urine of hypothyroid subjects. (Bastenie, 1939). Results of greater quantitative significance were obtained by De Robertis & Del Conte (1941) using a technique depending on the determination of the number of intracellular colloid droplets in the guineapig thyroid. A "cytological coefficient" (C_c) was derived from the formula:-

$$C_o = \frac{\text{Number of droplets} \times 10}{\text{Mean follicular diameter of six follicles}}$$

Test groups of four to six animals were starved for twenty-four hours prior to the assay; they were then given a single intrapericardial injection of 1 ml. of the test-substance and were killed thirty minutes later. The authors claimed that the lowest detectable dose was 0.0002 JSu/ml. (0.02 imu./ml.). Using a method depending on acetone precipitation, De Robertis (1948) was able to demonstrate the presence of TSH in serum from euthyroid subjects. Values in euthyroid subjects ranged from 0.0025 to 0.005 JSu/ml. (0.25 - 0.5 imu/ml.). The TSH-titre was found to be very low in four cases of untreated hyperthyroidism and in long-standing cases of myxoedema; in early cases of hypothyroidism the TSH-titre was approximately one hundred times higher than in the normal subjects, i.e. 0.5 JSu/ml. (50 imu/ml.). Del Conte & Vasena (1953) later described a simpler method of TSH assay by the "cytological coefficient"; by this test they found the normal levels of circulating TSH to lie in the range 0.001 - 0.01 JSu/2ml. of serum (0.05 - 0.5 imu/ml.)

A method based on calculation of the variation in relative amounts of epithelium, colloid and stroma present in thyroid sections was designed by Uotila & Kannas (1952). The tedious procedure of making histometric measurements was avoided, first by estimation of the areas covered by the three components and later by a simpler linear technique. A value, the "percentage of epithelium" (E%), was derived by measuring the distribution of the three components along six diameters of the section and calculating the proportion of epithelium as a percentage of the whole. Tala (1952) claimed a lower limit of sensitivity of 0.00001 JSu (0.001 imu/ml.) with this method. Kannas & Tala (1953) compared the

method of calculation of E% with a technique based on measurement of nuclear volume (Jacobij, 1931) and obtained comparable results.

2. Chick.

Jensen & Grattan (1940), Jorgensen & Wade (1941), and Cieresko (1945) independently defined "chick units" of thyrotrophic activity by assay techniques based on the method of Junkmann & Schoeller (1932). Using test-groups of three animals given daily injections for three to five days Jones (1939) attempted to demonstrate the presence of TSH in urine extracted in various ways. A series of tests performed on serum extracted by the method described by Fellinger (1936) yielded findings of an equivocal nature (Collard, Mills, Rundle & Sharpey-Schaefer, 1940). Although five daily injections were administered to test groups of four animals, in the course of which each chick received the equivalent of 15 ml. of whole blood, it was not possible to detect TSH in the serum of any of twelve euthyroid subjects investigated. A measurable concentration was demonstrated in one out of four cases of myxoedema and in four out of fifteen patients with thyrotoxicosis.

Rawson & Salter (1940) improved the reliability of the assay by using larger numbers of test-animals and by performing 100 cell-height measurements on each animal. The lower limit of sensitivity was quoted as an absolute value of 0.25 JSu (25 imu). This method was applied by Savoie (1952) to acetone extracts of urine. A positive response was obtained in nine patients with exophthalmos, five with spontaneous myxoedema and four out of fourteen euthyroid subjects. Savoie (1952) was unable to detect TSH in four cases of thyrotoxicosis investigated. The method described by Rawson & Salter (1940) was also applied in estimations of TSH levels in dog serum (Fraja & Martini 1953). These

workers claimed a lower limit of sensitivity of 0.2 imu/ml. and obtained a measurable response when serum from euthyroid animals was injected. Dobyns & Steelman (1953) investigated a method of differentiating between the thyroid-stimulating and exophthalmos-producing factors of pituitary extracts. Although it now appears unlikely that TSH levels in body fluids and exophthalmos are closely allied, their relationship to the clinical manifestations of thyrotoxicosis has been the subject of much controversy. TSH was assayed by three methods, gravimetric, histometric and by a technique dependent on chemical estimation of the thyroidal iodine content (Piotrowski, Steelman & Koch, 1953). Exophthalmos-producing substance (EPS) was assayed using Fundulus heteroclitus, the Atlantic minnow; the end point depended on the percentage increase in intercornecal distance. It was found that the TSH fraction could be obtained free of EPS, but that the EPS extract always retained some thyroid stimulating activity. These observations, although of considerable interest, do not however, exclude the possibility that abnormal pituitary function may be a contributary factor in the aetiology of both exophthalmos and thyrotoxicosis.

Dvoskin (1947) compared the reliability criteria of the histometric method with that of a modification of the intracellular colloid droplet method. He found that although the latter test appears to be highly sensitive, the specificity of the response is doubtful since droplet formation was shown to occur in response to injection of various substances other than TSH, such as histamine. A recent application of the method for estimation of TSH by increase in nuclear volume is described by Hess (1956), but the sensitivity obtained is not comparable

with that of the more familiar histometric techniques.

3. Rat.

Among the earlier studies on the level of TSH activity in body fluids are those of Herts & Oastler (1936) who obtained some "semi-quantitative" results by histological criteria in the young hypophysectomised male rat. A positive histological reaction was obtained with both serum and urine from myxoedematous patients, but no response was elicited by material from either thyrotoxic or euthyroid subjects. Herts, Means & Williams (1941) later demonstrated the presence of TSH in both serum and urine from patients with progressive exophthalmos. In the course of investigation of the changes induced in the thyroid cells by various anti-thyroid drugs, Lever (1950) showed that an increase in nuclear volume was induced by administration of extract of pituitary TSH in the young rat.

4. Amuran Tadpole.

The important part played by the thyroid gland in the course of development and metamorphosis of amphibian larvae was established by the classical experiments of Gudernatsch (1912), Allen (1916), Smith (1916), Hoskins & Hoskins (1917), and Smith & Smith (1922). Metamorphosis was found to be arrested as a result of removal of the thyroid and the same effect demonstrated indirectly after removal of the anterior pituitary anlage in the early neurula (Adler, 1914). The extreme sensitivity of the larval thyroid to changes in the level of trophic hormone to which it is exposed makes it an ideal test-animal for the assay of TSH. The larva of Rana pipiens was first used by Cuyler, Stimmel & McCullagh (1936) who studied the effect of administration of TSH on the time of eruption of the left fore-limb. Prior to this Spaul (1930) had demonstrated the effect

of induced metamorphosis after TSH administration in the axolotl.

Outstanding among the histometric methods is that of D'Angelo, Gordon & Charipper (1942), who used Rana pipiens larvae. This method had proved to be highly sensitive and reliable with a lower limit of sensitivity 0.002 JSu/ml. (0.2 imu/ml.). The technique makes use of the fact that starvation induces metamorphic stasis and thyroid atrophy in larvae at an early hind-limb stage, prior to the eruption of the fore-limbs. In this so-called "stasis" animal growth is arrested and the thyroid epithelium presents a uniformly squamous appearance. The main criteria employed for estimation of thyrotrophic stimulation are increase in acinar cell-height and in hind-limb length. At the time of its publication this method was shown to be between 60 and 100 times more sensitive than any of the available methods employing guineapigs or chicks. A similar method to that of D'Angelo et al. (1942) was used by Reineke & Turner (1942). When estimations are conducted on serum and other body fluids, the procedure has the further advantage that relatively small amounts of the test-material are required to treat large groups of animals.

D'Angelo et al.(1951) have applied their histometric method in studies on various vertebrate sera administering 5 daily injections of 0.05 ml. to animals starved 16 - 18 days prior to the injection period. They were able to detect TSH in some, but not all, of the euthyroid human sera tested, and quote a range of zero - 0.001 JSu/ml. (zero - 0.1 imu/ml.) in normal subjects. In 10 cases of hypothyroidism levels were higher than those in normal subjects and ranged from zero - 0.005 JSu/ml. (zero - 0.5 imu/ml.). High levels were also found in 3 cases of acromegaly. In two euthyroid infants aged 9 days and 3 months the titre was 0.8 imu/ml. and 0.6 imu/ml. respectively; no TSH activity could be detected in the

serum of a cretin of eleven years. (Di George, D'Angelo & Paschkis 1957). Di George et al. (1957) found a range of values from zero to 1.0 imu/ml. in serum from euthyroid subjects, with a mean value of 0.4 imu/ml.

Using a modification of the "stasis" tadpole technique, Simpkin, Starr & Hancock (1952) detected TSH activity in the serum of eight patients with hypothyroidism. No circulating TSH activity was detected in the serum of eleven euthyroid individuals, in two patients with panhypopituitarism, and in the majority of patients with thyrotoxicosis.

D'Angelo (1951) was able to demonstrate the presence of TSH in only a small proportion of patients with exophthalmos, but Asboe-Hansen, Iversen & Wichmann (1952), by the same method, obtained a positive response to nine out of ten sera from exophthalmic subjects. Asboe-Hansen et al. (1952) used the tadpole of Xenopus laevis and obtained comparable results by the histometric method and by the "E%" method of Uotila & Kannas (1952). As an alternative criterion of thyrotrophic stimulation they derived a "coefficient of metamorphosis" (C_m) from the formula:-

$$C_m = \frac{\text{Hind limb length}}{\text{Body length}}$$

This was applied in larvae in which the unstimulated hind-limb length was 3.5 - 4.5 mm. and body length less than 15 mm. The animals were regarded as suitable for use in the assay when C_m = 0.29. Any increase over this value at the end of the injection period was regarded as indicative of TSH stimulation. Thyrotrophic activity was not detectable in normal serum by the "E%" method for which the

"working range" is given as 0.0003 - 0.14 USP units. The lower limit of sensitivity in "high-titre" bloods, is quoted as 0.0015 USP units, an order of sensitivity somewhat inferior to that obtained by D'Angelo et al. (1942). Some of the individual values given for the activity present in these blood samples are considerably higher than any quoted by D'Angelo, and by most subsequent workers who conducted estimations on comparable material.

Deansley & Parkes (1945) and Dodd & Landgrebe (1953) also used the Xenopus tadpole for measuring TSH in body fluids. These Xenopus larvae are considerably smaller than the larvae of Rana pipiens and the maximum volume of fluid which can be administered parenterally is 0.03 ml. Dodd & Landgrebe (1953) observed a positive response to injections of serum from a patient suffering from thyrotoxicosis while Bowers & Segaloff (1957) obtained a response to 5 imu of TSH using Rana catesbiana as test-animal.

5. Other species.

Mason (1938) suggested the grass-snake, Tropidonotus natrix, as a possible test-animal for TSH assay. The resting thyroid shows a very uniform histological picture and, in animals maintained at 24°C, responds to injection of thyrotrophin by hyperplasia and colloid resorption. The concentration of TSH required to produce such a reaction was not specified. The winter sparrow, Passer domesticus, in which the gonads temporarily regress, was used by Wunder & Weibe (1940) as an alternative test-animal to the chick in a histometric assay and was shown to compare favourably in sensitivity with the mouse and guineapig.

Gorbman (1940) used test groups of 5 goldfish of body length 2 ins. in histometric assays on pituitary TSH extracts, regarding a 50% increase in average follicular cell-height as the minimal positive reaction after five daily intraperitoneal injections of 0.2 ml. In an assay similar

in design to that of Rawson & Salter (1940), Mercier-Parot & Tuchmann-Duplessis (1953) selected two species of newt, Triturus cristatus (syn. Molge) and Triturus palmatus. This choice depends on the fact that the thyroids of the newt undergo a cyclic variation in functional activity. In the resting state, between late summer and spring, the glands should be highly sensitive to thyrotrophic hormone. Mercier-Parot & Tuchmann-Duplessis (1953) were unable to reproduce D'Angelo's work on the Rana pipiens larva, but found that the histometric assay performed on the newt was approximately four times more sensitive than that using the hypophysectomised rat.

Comment.

Unfortunately, histometric assays are usually time-consuming, laborious and dependent on subjective interpretation. Other things being equal, these facts militate strongly against the use of such methods in clinical work. However, from the point of view of sensitivity and reproducibility, D'Angelo's "statis" tadpole method stands among the few which reach the required standards and which have yielded consistent and meaningful results.

C. Metabolic Methods and Chemical Estimation of Thyroidal Iodine

Little practical value can be attached to methods based on measurement of increase in basal metabolic rate after administration of TSH since such an indirect effect is only demonstrable after prolonged and intensive thyroid stimulation. Increase in BMR in the guineapig after treatment with TSH was measured by Schoedel (1933) and by Anderson & Collip (1934) who defined a unit of thyrotrophic activity as "the smallest amount which will induce a 20% increase in BMR in the hypophysectomised rat".

Estimation of thyroidal iodine depletion by chemical analysis was carried out by Cuyler et al.(1936) in the rat and by Pietrowski, Steelman & Koch (1953) in the day-old cockerel. The total iodine content

of the glands was determined by the method of Walaszek (Piotrowski & Koch, 1953) and the results calculated as micrograms of iodine per milligram wet weight of thyroid tissue. The minimal dose detected by this means was 0.02 USP units. Besides achieving only limited sensitivity, this type of chemical estimation involves a time-consuming and laborious procedure; such a method must therefore be regarded as suitable only for assaying extracts of relatively high TSH potency and is therefore of little value for studies on body fluids.

D. Radiometric Methods

The use of radioactive isotopes for the measurement of thyroid function made available several new techniques for assaying TSH: these were more rapid and often more sensitive than the methods already in use. Among the first to study the metabolic, as compared with morphological, effects of administered TSH using radioactive iodine were Leblond & She (1940) and Keating, Rawson, Peacock & Evans (1945). Use of radioactive indicators in the chick has been discussed by Wahlberg (1955). Radiometric methods have also been reviewed by Wolff (1951) and by Lamberg (1955) who arranged them according to their sensitivity and discussed their suitability for estimations on pituitary extracts or for detection of TSH in body fluids. The radiometric methods described in the literature may best be classified by dividing them according to the criteria employed to measure the effect of the hormone.

(a) Uptake of ^{131}I.

1. Guineapig.

Henry (1951) was able to demonstrate an increase of approximately 10% in ^{131}I-uptake in response to 0.75 "Heyl-Lacquer "units" after twice daily injections of 0.5 ml. given over a period of 3 days (25 imu/ml.).

Bloche-Michel & Henry (1952) made a thorough investigation of TSH levels in urine, the hormone being extracted by precipitation with alcohol and acetone. Parallel assays on urine and serum in the same subject yielded comparable results; for this reason the authors suggested that assays of TSH in urine might provide an index of the amount of hormone circulating in the body. The normal TSH excretion in a 24 hr. specimen of urine was 5 - 25 µg USP (0.25 - 0.75 imu.). In hypothyroidism the mean excretion value was found to be 3.25 imu/24 hrs. and in hyperthyroidism values ranged from 0.25 to 1.35 imu/24 hrs.

2. Chick.

Keating et al. (1945) stated that radiometric techniques were more efficient in measuring TSH than those depending on anatomical and cytological changes. These workers compared the response as measured by increase in acinar cell-height with that obtained by estimation of the ^{131}I content of a homogenate of the glands. They found that after injection of small amounts of TSH (0.125 - 0.25 imu) increase in accumulation of ^{131}I was proportionately greater than the degree of histological stimulation, but they observed a striking difference between the effect of TSH on ^{131}I accumulation and ^{131}I depletion in the thyroid. The latter response was found to occur largely within the first 24 hrs. following injection of TSH whereas iodine uptake became apparent only after 48 hrs.

The method described by Shellaberger (1954) was shown to be suitable for standardisation of pituitary extracts, but not for the detection of TSH in urine or serum. In the assay method described by Postel (1956) the end-point depended on ^{131}I-uptake by the chick thyroid after 3 daily subcutaneous injections of 0.1 ml. using test groups each containg 5 chicks. In order to compensate for the lack of sensitivity of the method, Postel

(1956) concentrated the TSH activity in serum by a procedure of zone-electrophoresis. The chief disadvantage of the test lies in the flatness of the dose-response curve over a dose-range of 5 to 50 imu/ml. In assays conducted on serum extracts, a TSH-inhibiting factor which affected the endpoint of the bioassay was encountered. The presence of such a factor has subsequently been demonstrated in serum by Adams (1958) and McKenzie (1958) and in urine by Greenspan & Lew (1958). The chemical nature of this inhibitor at present remains obscure.

3. Mouse.

A promising method using the mouse was described by Querido, Kassenaar & Lameijer (1953). Iodine uptake was estimated in iodocasein treated animals after 4 intraperitoneal injections given at 12 hr. intervals. The lowest dose of TSH detected was 20µg. USP provisional standard (20 imu). Lameijer (1956) conducted assays on serum concentrated by the method of Cohn et al. (1946). TSH was thus estimated in a serum-concentrate having a potency five times that of the untreated serum. By this means it was possible to demonstrate the presence of TSH in some, but not in all sera from euthyroid subjects. In a later paper Querido & Lameijer (1956) increased the sensitivity of their method to a level of 6µg USP (8.0 imu). In normal subjects, serum levels of TSH were in the range of 100-200 µg/100 ml. (1.0 - 2.0 imu/ml.). Normal levels were also found in a patient with severe progressive exophthalmic ophthalmoplegia. However, in a patient with post-operative myxoedema without exophthalmos a TSH-titre of 12,000 µg. USP/100 m. (120 imu/ml.) was found. On the basis of these limited observations the authors concluded that little or no relationship existed between exophthalmos on the one hand and serum TSH levels on the other.

4. Rat.

The method described by Querido et al. (1953) was employed by Overbeek, Fokkens, Querido, de Visser & Cannings (1953) using the intact rat, treated with iodocasein. It was found that when iodocasein was used the results were more consitent than those obtained with animals pretreated with thyroxine. The hypophysectomised rat has been employed by several workers including Ghosh, Woodbury & Sayers (1951), Brimblecombe (1952) and Levey, Cheever & Roberts (1956). Ghosh et al. (1951), using a Parke-Davis TSH preparation (13 JSu/mg.), found that the "working range" of the dose-response curve was obtained with total doses of 0.1 - 0.5 mg. per animal (33---165 imu/ml.). Estimation of the protein bound iodine showed that the levels in the blood followed the curve of the thyroid ^{131}I-content.

Levey et al. (1956) compared the sensitivity of hypophysectomised rats with rats treated with (i) thyroxine, (ii) thyroxine plus propyl thiouracil, and (iii) oral thyroid extract after a single intravenous injection of 0.2 ml. of thyrotrophin. Rats fed on thyroid extract were found to be most convenient to handle and responded linearly over a dose-range of 0.5 - 160 milli-units (2.5 - 800 imu/ml.). Reiss & Wyatt (1956) attempted to increase the precision of the assay method using littermate control in newborn rats. However, it was found that these young animals were relatively insensitive to TSH and were therefore unsuitable for use in clinical studies.

5. Anuran tadpole.

Radioactive tracers have been used to study amphibian development by Saxén, Saxén, Toivonen & Salimaki (1957^a, 1957^b), Gorbman & Evans (1941), Dent & Hunt (1952), (1957^a, 1957^b) and Shellaberger & Brown (1959). The

turnover of ^{131}I in normal, thiouracil and TSH treated Rana pipiens tadpoles was investigated by Money, Lucas & Rawson (1955). D'Angelo (1956) showed that the thyroid in larvae of Rana clamitans is capable of concentrating ^{131}I more effectively than radioactive ^{32}P. The minimal dose of exogenous TSH to which a significant increase in accumulation of ^{131}I was obtained was found to be 0.006 USP units (6 imu). This minimal effective dose was shown to be of the same order of magnitude with both single and multiple injections of TSH. A brief description of a similar study, using larvae of Xenopus laevis and estimating the increase of ^{131}I-accumulation by measurement of the activity in the isolated glands is given by Brown & Dodd (1956).

6. Other species.

The hypophysectomised gold-fish, Carassius auratus has been suggested as a possible test-animal for the assay of TSH by Chavin (1956). He was able to show that administration of TSH caused increase in thyroidal ^{131}I-accumulation up to 40.6% in hypophysectomised fish given a tracer dose.

(b) Discharge of ^{131}I.

1. Guineapig.

Adams & Purves (1955, 1957[a], 1957[b]) designed an assay method based on estimation of the increase in ^{131}I concentration in the plasma of guineapigs treated with thyroxine. The design of the assay is such that the need for a separate control group is eliminated, each animal acting as its own control. A blood sample is taken prior to intravenous injection of TSH and a second sample after an interval of three hours; the resultant discharge of ^{131}I is determined. Injection of TSH and measurement of the response can be repeated, on at least five consecutive

days, in the one animal. This ability to measure a succession of responses makes it possible to reduce greatly the number of test animals since the response to several dose-levels of the test-substance is determined in each animal during the course of a series of estimations. The lower limit of sensitivity, viz. 0.1 imu/ml., is such that TSH estimations can be carried out on untreated serum. However, Adams & Purves (1957) were unable to detect TSH activity in the serum of euthyroid subjects. One case of hypothyroidism was found to have a TSH-titre of 2.25 imu/ml. prior to treatment with thyroxine; following therapy the level fell to 0.25 imu/ml. The TSH levels in four cases of congenital hypothyroidism ranged from 1.0 to 2.5 imu/ml. A delayed response, possibly due to an abnormal form of TSH was obtained with serum from patients suffering from thyrotoxicosis (Adams, 1958).

2. Mouse.

McKenzie (1958), using mice, described a technique very similar to that of Adams & Purves (1955). The lower limit of sensitivity of this method is 0.5 imu/ml.; the degree of precision was reasonably satisfactory as indicated by a figure for the index of precision (λ) of 0.24. McKenzie (1957, 1958) found that TSH activity in serum travelled with the γ-globulins on electrophoresis and, using the method of Cohn et al. (1956), showed that the activity was confined to fraction II. The latter observation is at variance with the findings of Queride & Lameijer (1956) who claimed that TSH occurred in a number of fractions, namely II, III, and IV-4.

McKenzie (1958) detected TSH in the serum of six patients with myxoedema at a concentration 0.12 - 0.64 imu/ml. In a later paper, he described a delayed, abnormal response, similar to that encountered by Adams (1958), obtained after injection of serum from patients exhibiting thyrotoxicosis.

3. Chick.

An *in vivo* counting technique has been applied by two groups of workers, Gilliland & Russel-Fraser (1953) and Bates & Cornfield (1957). The latter used chicks treated with thyroxine and propyl thiouracil and found that a linear relationship existed between the logarithm of the dose and the degree of thyroidal iodine depletion over a dose range of 1.5 - 15 imu of TSH. The index of precision varied between 0.20 and 0.25. The authors suggested that a crossover design could be used, similar to that described by Adams & Purves (1955).

In the method described by Gilliland & Strudwick (1956), groups of 10 chicks, pretreated with thyroxine for 3 days, were used. The limit of sensitivity was 0.15 imu.; the precision of the method was unsatisfactory, as indicated by a figure for λ of 0.4 - 0.5. Determination of the radioactivity in the thyroid region was carried out *in vivo* immediately before and 48 hrs. after injection of the test-substance and the increase in percentage discharge by the treated groups in excess of that lost by the controls provided a measure of TSH activity. In the serum of euthyroid individuals these workers found that the TSH activity was approximately 0.16 imu/ml. They failed to obtain any response in cases of long established myxoedema. These findings appear to confirm the earlier observations of Emmerson & Cutting (1938) and De Robertis (1948) and support the view that pituitary secretion of TSH may decrease with time, and perhaps ultimately fail.

(c) Uptake of ^{32}P.

1. Guineapig.

Borell & Helmgren (1949) estimated the increase in uptake of ^{32}P in response to injected TSH in the immature guineapig. In comparison

with the histometric technique, they found that by use of ^{32}P the dose-range was extended and, for a given dose, the resulting increase in ^{32}P-uptake was five times greater than the increase in cell-height. The authors considered that ^{32}P-uptake in response to administered TSH is closely related to increase in cell-height of the follicular epithelium.

2. Chick.

A comparison of the thyroidal ^{32}P-uptake response with increase in "[illegible]" was made by Lamberg (1953). The smallest detectable dose of TSH was found to be 0.005 JSu (0.5 imu.) using day-old chicks in groups of twenty. Contrary to the conclusions of Borell & Holmgren (1949), Lamberg, Wahlberg & Olin-Lamberg (1955) were unable to demonstrate a close relationship between the ^{32}P-uptake by the glands and the increase in cell-height. Crooke & Matthews (1953) described a method for determination of TSH involving measurement of ^{32}P-uptake. In their view the method is specific for TSH, no response being elicited by administration of either adrenocorticotrophin or human chorionic gonadotrophin. A measurable response was obtained after injection of an ultrafiltrate of urine from a patient with exophthalmic ophthalmoplegia, the equivalent of 15 ml. untreated urine being administered per chick.

The day-old cockerel has also been used by Greenspan, Kriss & Moses (1954, 1956). These workers measured increase in thyroidal ^{32}P-uptake 6 hrs. after a single intracardiac injection of 0.1 ml. of the test-substance and found that the "working range" of the dose-response curve lay between 0.00025 and 0.005 USP units (2.5 - 5 imu/ml.); the mean index of precision for their series of assays was 0.34. Some preliminary experiments indicated that the method was suitable for detection of TSH in an ultrafiltrate of a 24 hr. specimen of urine from euthyroid subjects.

In a more recent paper Greenspan & Low (1958) draw attention to the limitations of this assay method as applied to blood and urine; they showed that the ultrafiltrate residue of urine contains a factor distinct from TSH which stimulated uptake of ^{32}P by the thyroid of the chick and which interferes with the effect of TSH on thyroidal ^{131}I depletion.

3. Rat.

A method similar to that of Crooke & Matthews (1953) was described by Dedman, Stuart-Mason, Morris & Morris (1953), who measured the response to TSH in the hypophysectomised rat. Although the authors considered increase in ^{32}P-uptake to be a direct effect of administered TSH they demonstrated a lack of correlation in the pattern of ^{32}P-uptake and ^{131}I-uptake by the thyroid glands in response to exogenous TSH.

Comment.

The use of radiometric criteria has brought a further improvement in sensitivity and rapidity of TSH estimations although, with the exception of the methods described by McKenzie (1958) and Gilliland & Strudwick (1956), none of the techniques employed are capable of detecting TSH in untreated serum from euthyroid subjects. At the time of writing, few attempts have been made to assess the results obtained by the various methods according to recognised statistical criteria. Where reliability criteria have been quoted, e.g. in papers by Gilliland et al.(1956) Greenspan et al. (1956) and McKenzie (1958), the degree of precision has not always been entirely satisfactory.

Although Borell & Holmgren (1949) obtained good agreement in the response of epithelial hypertrophy and ^{32}P-uptake after administration of TSH, Lamberg et al. (1955) found that the two endpoints were not directly comparable. Measurement of uptake of ^{32}P must be regarded as an indirect

method of estimation of thyrotrophic activity and, as such, less satisfactory than the direct estimation of either ^{131}I-depletion or accumulation.

The data presented by Gilliland & Strudwick (1956), McKenzie (1958) and Greenspan et al.(1958) agree with the findings of Di George et al. (1957) using the histometric method of D'Angelo et al. (1942) that euthyroid levels of TSH in serum are generally below 0.5 imu/ml. Further work on fractionation methods is indicated in view of the difficulties encountered in attempts to concentrate TSH from serum (Querido et al. 1956). The presence of a factor which interferes with the action of TSH in both serum and urine is of considerable theoretical and clinical interest. The fact that such a factor is present in urine extracts prepared by acetone precipitation and by ultrafiltration suggests that it is not an artefact introduced by the method of treatment of the raw material. However, its precise role in thyroid physiology can not be determined until such time as it has been separated from TSH in either serum or urine.

E. In Vitro Methods

A strong argument in favour of in vitro assay techniques as compared with assays in the intact animal lies in the fact that the test-substance is placed in immediate contact with the isolated tissue on which it exerts its effect.

Galli-Mainini (1941, 1942) described the first in vitro method for measuring TSH, based on estimation of the change in respiratory quotient (RQ) of surviving guineapig thyroid tissue slices exposed to serum from patients exhibiting various thyroid disorders. Serum from patients suffering from acromegaly, untreated myxoedema and thyrotoxicosis with associated exophthalmos gave a positive response; no change in RQ occurred after exposure to serum from cases of thyrotoxicosis unassociated with exophthalmos or from cases of myxoedema

after therapy. Junqueira (1947) demonstrated an increase in the number of intracellular colloid droplets in surviving thyroid tissue of both rat and guineapig 30 mn. after addition of TSH to the nutrient medium in which the tissue was incubated.

Renewed interest in this field has been aroused by the development of <u>in vitro</u> techniques dependent on radiometric criteria (Bakke & Lawrence 1956, Bakke, Heidsman, Lawrence & Wiberg 1957, Bottari, 1956, 1957, 1958, Florsheim, Moskowits, Schwartz & Morton 1957). In its present form, the method of Bottari (1958), depending upon estimation of percentage increase in discharge of ^{131}I by surviving guineapig thyroid tissue, gives a linear dose-response curve spanning a range of sensitivity of 0.1 - 10 imu/ml. of TSH administered (Bottari & Donovan 1958). The tissue from one animal is evenly distributed between eight culture tubes for incubation with ^{131}I. Of these, three tubes are used to construct a dose-response curve for the standard preparation and the remaining five for assay of test-samples. The whole procedure is completed within 24 hrs. and the sensitivity is such that the normal levels of TSH in the serum of euthyroid subjects can be readily determined. The method is reasonably precise as indicated by a mean figure for λ of 0.12.

Bottari (1958) found that in 120 euthyroid men between the ages of 20 and 60 the mean TSH level in serum was 0.22 imu/ml. In women during reproductive life TSH levels were higher than those in normal men and in post-menopausal women, the mean figure being 0.37 imu/ml. Increased blood levels were also found in patients with untreated myxoedema.

The test first described by Bakke & Lawrence (1956) depended on estimating ^{131}I discharge using ox thyroid slices. The limit of sensitivity is among the lowest recorded, a significant response being obtained with as

little as 0.008 imu/ml. of medium; the working range of the dose-effect curve was relatively limited, extending from 0.008 to 0.04 imu/ml. Baldes et al. (1957) later described an in vitro technique based on a weight increase response in ox thyroid slices. The assay was shown to be simple, economical, specific and sensitive to less than 0.01 imu/ml. The precision of the method compares favourably with that of other current methods, with an average value for λ of 0.28.

Flersheim and his colleagues (1957) employed an assay procedure dependent upon the incorporation of ^{32}P into the lipoid fraction of surviving beef thyroid tissue. The limit of sensitivity of the technique was 2 milliunits of TSH and the index of precision was 0.22.

Comment.

Besides attaining a high degree of sensitivity these in vitro techniques are in general simple and rapid to perform. For example, in the method described by Bottari & Donovan (1958) the assay procedure is completed within a few hours. Such methods have the further advantage that the necessity for using large numbers of animals does not arise. In addition, the problem of variability in response of individual test-animals is not encountered since a series of estimations are carried out on tissue provided by one individual. It should, however, be emphasised that in order to obtain reliable results by this method the animals must be kept under rigidly controlled conditions.

PART II

HISTOMETRIC METHODS

PART II

HISTOMETRIC METHODS

I. INTRODUCTION

1. The need for a biological assay technique.

Little is known concerning the chemistry of TSH; there is, therefore, no possibility, as yet, of measuring it by chemical techniques. Consequently, as has already been stated in the survey of the literature, there is a need to establish a biological assay method; this is needed both as a research tool and also in routine chemical investigations.

The level of TSH circulating in the body assumes importance indirectly through its control of thyroid function; besides numerous lesser effects in the body, the thyroid hormone regulates the basal metabolic rate. Thus, it becomes apparent that a method for estimation of TSH in pituitary tissue and in body fluids is an essential complement to assays of circulating thyroid hormone.

From the clinical aspect, the need for a reliable and sensitive method for assay of TSH lies in the fact that abnormalities of thyroid function constitute a major cause of illness in man. A method of the required sensitivity, reliability and technical simplicity would contribute information concerning the aetiology of both hypo- and hyperthyroidism. It would thus be of assistance in the determination of whether a particular thyroid condition is primary or secondary in origin. Further, it would enable us to study the variation in TSH levels in euthyroid men and women in different age groups, the mechanism of control of thyroid metabolism in infancy and the changes occurring during the course of pregnancy. In addition, such a method, used in conjunction with estimations of gonadotrophins and oestrogens, would have an application in the investigation of

the effectiveness of pituitary inhibitor drugs. It does not at present appear that earlier views postulating a causal connection between TSH and exophthalmos were well founded, though this is another aspect of the subject which requires further study using a sensitive assay technique.

2. Sensitivity and specificity.

The question of sensitivity has already been discussed in some detail. The histometric technique has been shown to be among the few methods which reach the required standard of sensitivity for estimation of TSH in untreated serum from euthyroid subjects. The method described by D'Angelo, Gordon & Charipper (1942) has continued to yield meaningful results over a period of years; moreover the results obtained by this method compare favourably with those published more recently using radiometric techniques. It is apparent from these more recent findings that some of the methods employed in the past have not been specific for TSH. In this respect, the use of a method dependent on measurement of histological changes induced in the thyroid is advantageous as compared with methods in which the end-point may be regarded as an indirect rather than a direct effect of TSH.

3. Histometric techniques.

The following histological criteria have been held by various workers to yield a reliable measure of thyroid stimulation:-

1. Measurement of increase in cell height.
2. Measurement of increase in nuclear volume in the thyroid cells.
3. Measurement of increase in mitotic activity in the thyroid cells.
4. Measurement of increase in the number of intracellular colloid droplets.
5. Measurement of increase in the percentage of epithelium.
6. Measurement of increase in the percentage of colloid.

The simplest of these is measurement of the acinar cell height from the basal membrane of the follicle to the inner wall of the cell.

It is clear that TSH influences not only thyroid cell-height, but also the number of cells and the amount of colloid present. Lever (1950) has discussed a method of utilising histological material by which not one, but three of the characteristics of the follicle, which are altered as a result of thyroid stimulation, are measured in conjunction with one another to give a more accurate determination of change in thyroid function. The sensitivities of four of the above histological methods has been compared by Lever & Vlijm (1955).

Measurement of the increase in the number of intracellular colloid droplets as described by de Robertis & del Conte (1941) has since been shown to be non-specific (Dvoskin, 1947), as has also measurement of increase in mitotic activity in the acinar cells. Both these effects can be induced by a number of substances other than TSH.

The time required for the preparation and subsequent examination of histological material is a major obstacle to routine application of an assay based on histometric criteria. Several attempts have been made to circumvent this difficulty. A projection method for the determination of the percentage of epithelium has been employed by Uotila & Kannas (1952) and by Asboe-Hansen et al. (1952). The use of planimetry has been explored by van Eck (1940), Stein (1940) and Cordin & Herlant (1956). The subjective interpretation of results may be reduced by using projection methods such as these, as compared with microhistometric methods. However, certain other errors are introduced as a result of magnifying the image of the section.

4. Tadpole techniques.

The rationale on which the choice of the anuran tadpole as a test-animal for TSH assay is based has been discussed above (page 9). The tadpoles most extensively used in thyroid studies have been those of Rana pipiens and other Rana species, but it has not been possible to breed Rana pipiens larvae under laboratory conditions. The tadpoles must therefore be collected in the field and work employing these animals is thus subject to certain seasonal limitations, although the larval life is comparatively long, approximately 2 years.

On the other hand, use of the tadpoles of Xenopus laevis has the advantage that the adult toads are easily maintained in the laboratory and larvae can be obtained throughout the year by means of artificially induced ovulation (Landgrebe & Purser, 1941, Landgrebe & Samson, 1944). As compared with other laboratory animals, both the adult toads and the larvae can be maintained at low cost and their husbandry is relatively easy.

The procedure followed by D'Angel et al. (1942) and by subsequent workers using their technique has been outlined above (page 10.). When assays are to be conducted on serum or other body fluids of which only a limited volume is available a further advantage of the tadpole lies in the fact that only a small amount of the test-substance is needed to treat large groups of animals.

5. Present studies.

It was decided, in the present investigations, to determine the range of sensitivity of the histometric method using Xenopus larvae reared in the laboratory and to asses the practicability of the method

in clinical research.

II. MATERIAL AND METHODS

1. Husbandry of Animals.

It has been demonstrated that a supply of Xenopus larvae in all stages of development can be maintained in the laboratory throughout the year (Landgrebe & Purser, 1941, Landgrebe & Samson, 1944, Deansley & Parkes, 1945). The conditions of feeding and temperature selected for routine rearing purposes have been based on findings concerning the effect of diet and temperature on thyroid function which are described elsewhere (Part III) and apply to animals used in both radiometric and histometric studies. The aim, in both cases, being to maintain a supply of large, metamorphically retarded animals suitable for handling and for injection and in which individual variation is reduced to a minimum.

Fertilised eggs were obtained by artificially induced ovulation. Chorionic gonadotrophin was injected into the dorsal lymph sac of both the male and the female toad; the female received approximately 120 International Units and the male half this dose. The injected toads were then isolated in a 5 litre beaker containing a closely fitting crystallising basin, inverted to form a platform such that the eggs collected round the sides, out of reach of the toads. The toads were separated after 48 hours and the eggs hatched in running water at a constant temperature of 20 - 22°C.

The young larvae were transferred to a shallow aquarium and fed on a suspension of dried liver powder; later, when of sufficient size to withstand individual handling, they were placed in jars each containing 10 animals in 700 ml. of water. In the jars the larvae were reared on a 50/50 mixture of liver powder and dried yeast in suspension, 100 mg/3days, under constant illumination and at a temperature of 18°C.

2. Experimental.

(a) Selection of experimental animals.

Wherever possible animals were used in test-groups of at least 10 in number. This, however, presented a considerable problem, since to procure a uniform test group a very much larger population must be reared and maintained, of which at least 50% of the animals are ultimately discarded as unsuitable. Although supplies of fertilised eggs can be obtained at all seasons, the number of larvae which can be reared is dependent, to some extent, on the technical assistance available. The limitations imposed by this consideration made it necessary, in performing the preliminary assays of TSH in serum samples to use test-groups of fewer than 10 animals.

The method described by D'Angelo, Gordon & Charipper (1942) makes use of the fact that starvation induces thyroid atrophy and metamorphic stasis in tadpoles at early hind-limb length stages. D'Angelo & Gordon (1950) quote 5 mm. as the maximum hind-limb length at which "stasis" can be induced by inanition in larvae of Rana pipiens. It was found, however, in both histometric and radiometric studies, that in the Xenopus larvae this critical stage, at which starvation ceased to cause arrested metamorphosis and instead induced an acceleration of the changes, occurred at a hind-limb length of 8 - 10 mm.

To obtain a uniform group in preparation for an assay, e.g. 50 larvae,

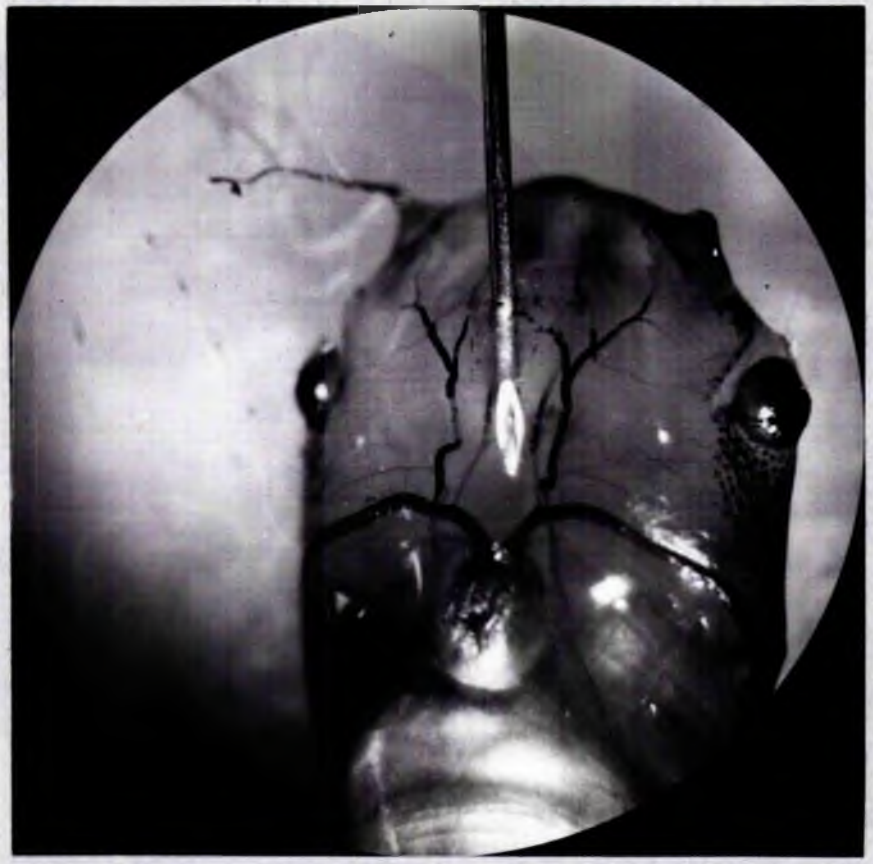


Plate I.

Injection technique in the Xenopus tadpole; the needle is shown in position in the submental lymph space.

Magnification x 50

for 5 test-groups, the animals from 10 jars were pooled and the smallest and largest larvae were discarded until a uniform group of the required number was obtained. The hind-limb lengths of these larvae were measured using a pair of fine-pointed Vernier callipers and holding the animal on a piece of damp cotton-wool. The tadpoles were then distributed into groups.

(b) Method of Starvation.

The "stasis" condition was induced by a starvation period of eight days prior to the injection period. The water was changed each day to avoid growth of micro-organisms which might serve as a source of food. It was found that viability was greatly reduced if the animals were starved in distilled water; they were less able to withstand the handling necessary during the subsequent injection period. All experimental procedures were therefore carried out using tap water.

(c) Method of injection.

The sub-mental lymph space is the most suitable site for injection in Xenopus larvae, using the interhyoideus muscle as a seal. This is the only possible site in larvae of hind-limb length 4 - 8 mm., the stage of development at which they were most frequently used. The larvae were placed ventral side up on damp cotton wool on the stage of a binocular dissecting microscope and the test-substance injected into the submental lymph space using an "Agla" micrometer harness with a ground glass syringe Plate.I. The "Agla" harness makes it possible to administer a volume of 0.005 ml. with a good degree of accuracy. A maximum volume of 0.02 ml. can be injected into the submental lymph space without leakage occurring, but mortality of the test-animals is greatly increased with this amount. Accordingly, where a series of daily injections was employed, 0.01 ml. or

0.02ml. was given.

In larger larvae, of hind-limb length > 8 mm. it is possible to use an intraperitoneal injection route, with the leg muscle acting as seal. This was adopted in some of the radiometric investigations. In the series of assays performed, no fixed injection schedule was employed but wherever possible a total of 6 daily injections was given. In instances where smaller animals were used, which did not withstand handling well, the injection period had to be reduced to 5 or 4 days.

(d) Preparation of solutions for injection.

Standard solutions.

The International Standard Thyrotrophin (potency 74 imu/mg.) supplied by the Department of Biological Standards, Millhill, was used as a reference substance throughout the experimental work described below. The Standard material, supplied in tablet form, was dissolved in 0.75% saline at a concentration of 50 imu/ml. This constituted the stock solution and was stored at 2°C. More dilute solutions for injection were made up daily from this stock solution.

The keeping qualities of TSH in solution have not been examined in detail, but the activity has been shown to fall off rapidly at low concentration. The stock solution made up at 50 imu/ml. has been found to be stable when stored in the cold.

In previous work on TSH assay methods confusion has arisen through lack of standardisation and expression of results in "animal units". In more recent investigations of serum TSH levels, findings have been expressed in imu/ml. of serum (Gilliland & Strudwick, 1956, Di George, D'Angelo & Paschkis, 1957, McKenzie, 1958, Bottari, 1958). This method of expressing the concentration of TSH in the standard solutions administered has therefore

been adopted throughout assays performed.

Serum.

20 ml. samples of blood were collected from the patients and allowed to stand for 24 hrs. at 2°C in a clean centifruge tube. They were then centrifuged for 20 min. at 2,000 rpm. and the serum pipetted off and stored in the deep freeze in a universal container. The serum was allowed to thaw at room temperature prior to injection.

(e) Histological techniques.

24 hrs. after the last injection the test animals were killed and fixed by immersion in alcoholic Bouin's solution for 6 hrs. They were then transferred to 70% alcohol for a further 6 hrs. The lower jaw, containing the thyroids, was then dissected off immediately anterior to the heart and allowed to stand overnight in 70% alcohol before being taken up through a series of alcohols, cleared in xylol and embedded in paraffin according to the schedule in Table I.

TABLE I

HISTOLOGY SCHEDULE

Bouin's solution (alcoholic)	6 hrs.
70% alcohol 1.	6 hrs.
70% alcohol 2.	overnight.
80% alcohol	1 hr.
90% alcohol	1 hr.
95% alcohol	1 hr.
Absolute alcohol 1.	1 hr.
Absolute alcohol 2.	1 hr.
Absolute alcohol-xylol 50/50 v/v	30 mins.
Xylol	1½ hrs.
Xylol-paraffin 50/50 v/v	25 mins.
Paraffin 1.	20 mins.
Paraffin 2.	20 mins.
Paraffin 3.	25 mins.
Embed.	

Serial sections of the thyroid, cut at 5μ were stained with Ehrlich's haematoxylin, counterstained with alcoholic eosin and mounted.

Measurements of the thyroid acinar cell-heights were made using a Watson micrometer eye-piece (calibration:- 3 micrometer divisions ≡ 1μ). The cell-height measurement taken constitutes the depth from the basal membrane of the follicle to the inner border of the cell. Thus, only cells of which both borders are clearly defined can be used and it is necessary to select these cells deliberately. The problems resulting from making selective measurements in this way are discussed in detail below.

Thirty micrometer readings were made on each animal and the mean value obtained was converted into μ. The results were then expressed as a percentage increase in mean cell-height (MCH) over the control cell-height.

(f) Design of assay.

To establish a working dose-range, histometric assays were carried out using 3 dose-levels of TSH and a saline injected control group. This was reduced to two dose levels of standard in assays performed on serum. A 4-point assay design, incorporating two dose-levels of standard and two of the unknown with an equal interval between in both cases has been shown to be the most useful structure in bio-assays of this kind since it gives the smallest error for a given number of animals and simplifies the process of calculating the limits of error and probability for the results. By a test of parallelism the validity of the assay can also be deduced. Using 2 or 3 dose levels of standard and one of the unknown a less satisfactory result is obtained and there is no means of testing for validity (Gaddum, 1953). Since the experiments to be described took the form of a trial of the application of the histometric method in tests on serum the latter design was used in spite of its limitation, for two reasons:-

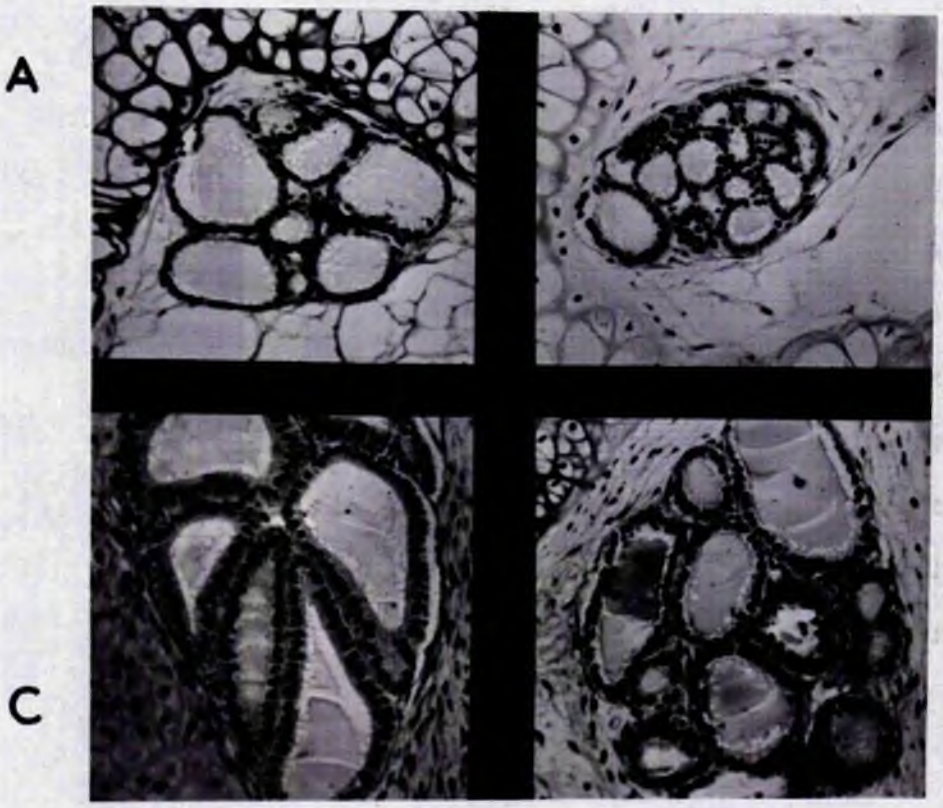


Plate II.

Histological stimulation in the thyroid of the Xenopus tadpole in response to injection of TSH:-

A. Saline injected control.
B. TSH injected (10 imu/ml.).
C. TSH injected (50 imu/ml.).
D. Serum injected; donor suffering from post-operative myxoedema.

1. That little information has been accumulated concerning the actual level of concentration of circulating TSH and much of this is equivocal.

2. That the expected order of concentration of circulating TSH in euthyroid subjects and in some thyroid disorders is extremely low. Thus, the accepted method of preparation of a second dose-level by simple dilution of the test-substance could not be applied in such cases.

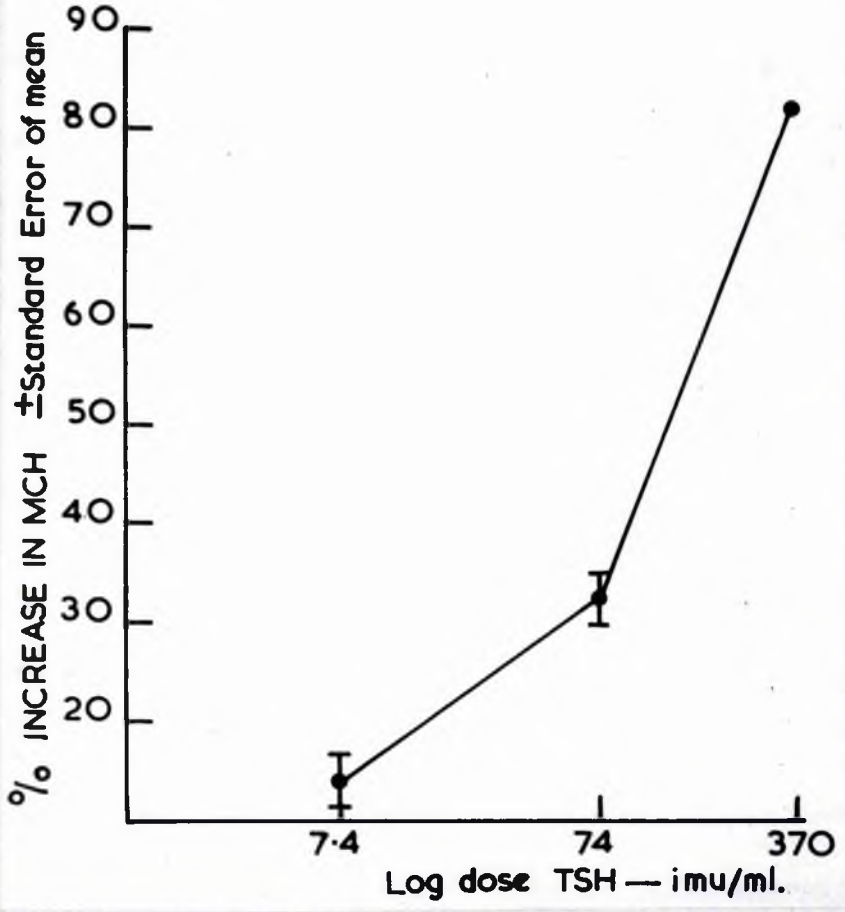
The assays performed on serum are therefore best described as "semi-quantitative". Subjects were selected in whom a high titre of TSH was to be expected on the basis of the generally accepted theory of thyroid-pituitary inter-relationship i.e. cases of myxoedema in which a low level of circulating thyroxine would be expected to stimulate an increased secretion of thyrotrophin by the pituitary. The findings of others also indicate that a higher level of TSH is demonstrable in the blood of most individuals suffering from a hypothyroid state which is not of pituitary origin. The type of histological picture obtained after injection of serum having a high TSH-titre is illustrated in Plate II.

III ASSAY OF PITUITARY THYROTROPHIN

1. International Standard Thyrotrophin.

(a) A pilot assay was performed for the purpose of establishing a suitable dose-range on which to carry out full-scale assays.

FIG. 1.


% INCREASE IN MCH ±Standard Error of mean
90
80
70
60
50
40
30
20
7·4
74
370
Log dose TSH — imu/ml.

Experimental routine:-

Number of animals = 20

Starvation period = 8 days

Injection schedule = 0.01 ml./day/5 days.

RESULTS.

TABLE II

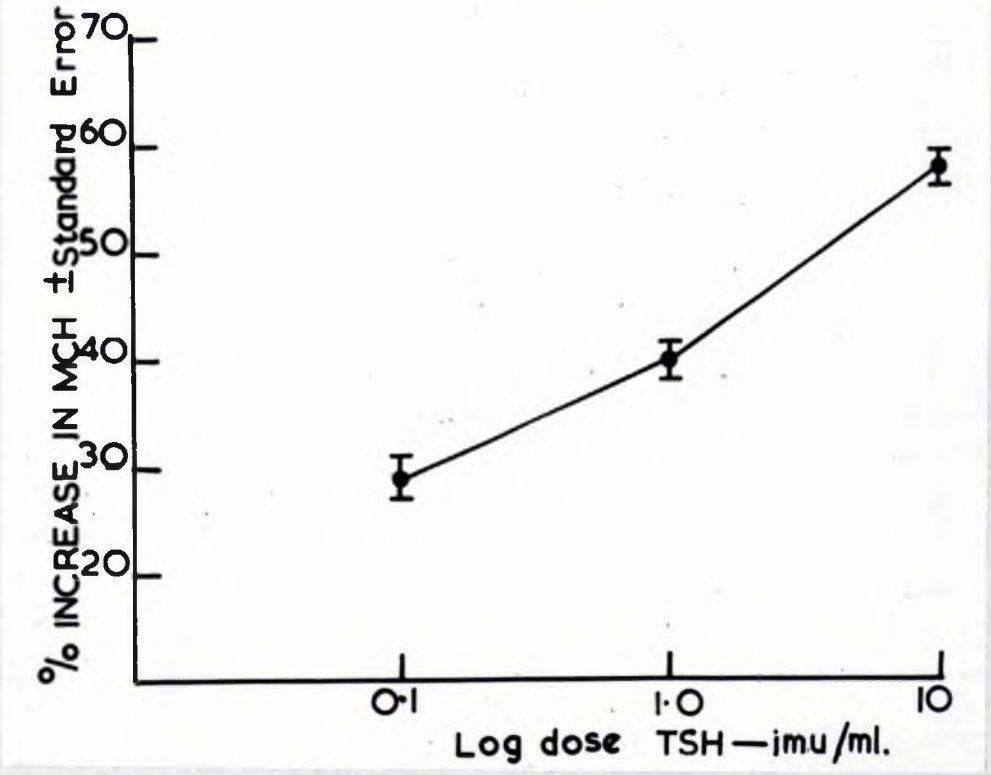
Dosage TSH imu/ml.	370	74	7.4	Control
Number of animals	2	5	5	5
Mean hind limb length (mm.)	6.3	5.4	5.8	4.9
MCH of individuals within group. (μ)	13.7 14.2	10.16 10.50 10.13 10.08 10.26	9.8 9.13 8.26 8.53 8.15	7.8 7.76 8.06 7.83 7.76
MCH of group (μ).	14.0	10.21	8.78	7.70
S.E. of mean	-	± 0.32	± 0.309	± 0.174
S.D. of mean	-	± 0.715	± 0.690	± 0.388
Increase over control	6.3	2.51	1.08	-
% Increase over control	81.8%	32.6% ± 3.13%	14.0% ± 3.52%	

The highest dose-level of TSH used (370 imu/ml.) was found to be toxic; only two animals survived the complete injection schedule. Gross metamorphic effects were apparent and there were marked indications of histological stimulation in the two animals on which cell-height measurements were made.

(Table III Figure 1.). In view of this distinctly unphysiological response obtained and the fact that such high titres of TSH would not be expected in any untreated body fluids to be tested, the upper limit of working dose-range was taken to be in the region of 74 imu/ml.

(b) The lower part of the dose-range was then explored in greater detail to establish the lower limit of detection. The results of a typical assay using larger groups of animals are shown in Table II and in Figure 2.

FIG. 2.


% INCREASE IN MCH ± Standard Error
70
60
50
40
30
20
0·1
1·0
10
Log dose TSH — imu/ml.

Experimental routine:-

Number of animals = 56

Starvation period = 8 days.

Injection schedule = 0.02 ml/day/6 days.

These results were analysed by the conventional methods of statistics to show the degree of precision and discrimination obtained in an assay performed on this scale.

TABLE III

Histometric Assay of International Standard TSH

(Observer A)

	x	x'	x''	x'''
Dosage TSH imu/ml.	10.0	1.0	0.1	Control
Number of animals	10	13	14	11
Mean hind-limb length. (mm.)	2.9	2.5	2.7	2.0
MCH of individuals within group (μ)	9.64 9.35 9.92 9.54 9.46 9.67 8.87 10.36 9.07 9.95	8.76 9.01 8.05 7.91 8.58 8.58 8.61 8.10 8.55 8.12 8.01 8.28 8.44	7.98 9.23 7.45 7.78 8.00 7.76 7.35 7.61 8.00 7.76 7.74 7.52 7.51 7.31	6.36 6.18 6.04 6.38 6.38 6.02 6.11 5.85 5.37 5.63 6.11
MCH of group (μ)	9.49	8.44	7.78	6.04
S.E. of mean	±0.15	±0.108	±0.126	±0.094
S.D. of mean	±0.474	±0.389	±0.471	±0.284
Increase over control	3.45	2.40	1.74	
% Increase. * Standard error	57.4 ±1.59	39.8 ±1.28	28.8 ±1.62	

Calculation of Results.

The results obtained were evaluated according to the generally accepted methods of statistical analysis applied in this type of bio-assay.

(i) The index of precision (λ) was calculated for the three dose-levels of International standard from the formula $= \frac{b}{s_D}$ where:-

"b" the slope $= \frac{\bar{x} - \bar{x}''}{2} = \frac{9.49 - 7.78}{2} = \frac{1.71}{2} = \underline{0.855}$

s_D, common standard deviation for the 3 groups x, x', x", was obtained using the formula:-

$$S^2 x, x', x'' = \frac{S(x^2) - \bar{x} S(x) + S(x'^2) - \bar{x}' S(x') + S(x''^2) - \bar{x}'' S(x'')}{N + N' + N'' - 3}$$

$$\text{Then } s_D = \sqrt{\frac{S^2 x\, x'\, x''}{N} + \frac{S^2 x\, x'\, x''}{N'} + \frac{S^2 x\, x'\, x''}{N''}}$$

Thus $s_D = \underline{0.3255}$

$$\therefore \lambda = \frac{b}{s_D} = \frac{0.855}{0.3255} = \underline{0.38}$$

(ii) Discrimination between the several dose-levels was calculated using the students "t" test for significance where:-

$$t = \frac{\bar{x} - \bar{x}'}{s_D}$$

and the degree of freedom $n = N + N' - 2$

To obtain s_D, $S^2 x, x'$ was calculated as above, then

$$s_D = S x\, x' \sqrt{\frac{N + N'}{NN'}}$$

The values for p calculated were:-

$x - x'$ (10 imu/ml. and 1.0 imu/ml.).

$n = 23$

$s_D = 0.1943$

$t = 5.526$

$\therefore \quad p = < 0.001$

$x' - x''$ (1.0 imu/ml. and 0.1 imu/ml.).

$n = 25$

$s_D = 0.07758$

$t = 8.5073$

$\therefore \quad p = < 0.001$

$x'' - x'''$ (0.1 imu/ml. and control)

$n = 23$

$s_D = 0.07431$

$t = 2.208$

$\therefore \quad p = 0.05 - 0.02$

Values for λ of 0.2 are acceptable for this type of bio-assay. The figure obtained, $\lambda = 0.38$, may therefore be taken as indicative of a reasonable degree of precision in this case. Where the value for p is less than 0.05 the difference between the means should be regarded as significant.

Thus, the results obtained demonstrate that a significant increase in cell height occurs in response to injection of 0.1 imu/ml. of standard TSH when test-groups of more than 10 animals are employed, and 0.1 imu/ml. should be regarded as the lower limit of detection.

Reproducibility of micrometer readings.

A considerable subjective element is involved in taking measurements using an eyepiece micrometer. Only cells of which both the internal and external borders are clearly defined can be used, which necessitates deliberate selection of the cells to be measured. Variation in cell-height is observed in any individual tadpole, including the control animals in which the epithelium presents an overall squamous appearance. Since it is not possible to measure a true random sample of cells because of these limitations, it was determined that a method of deliberate selection should be adopted. Thus for every "small" cell measured a correspondingly "large" cell was also selected, ensuring as nearly as possible an even distribution of high and low readings to cover the whole range of cell heights presented in any one animal.

To test the reproducibility of results obtained by this method of deliberate selection, a duplicate set of readings were made by a second observer (Observer B) for comparison with those already recorded in Table III (Observer A). The readings obtained by Observer B are recorded in Table IV. The two sets of results are compared in Figure 3.

FIG. 3.

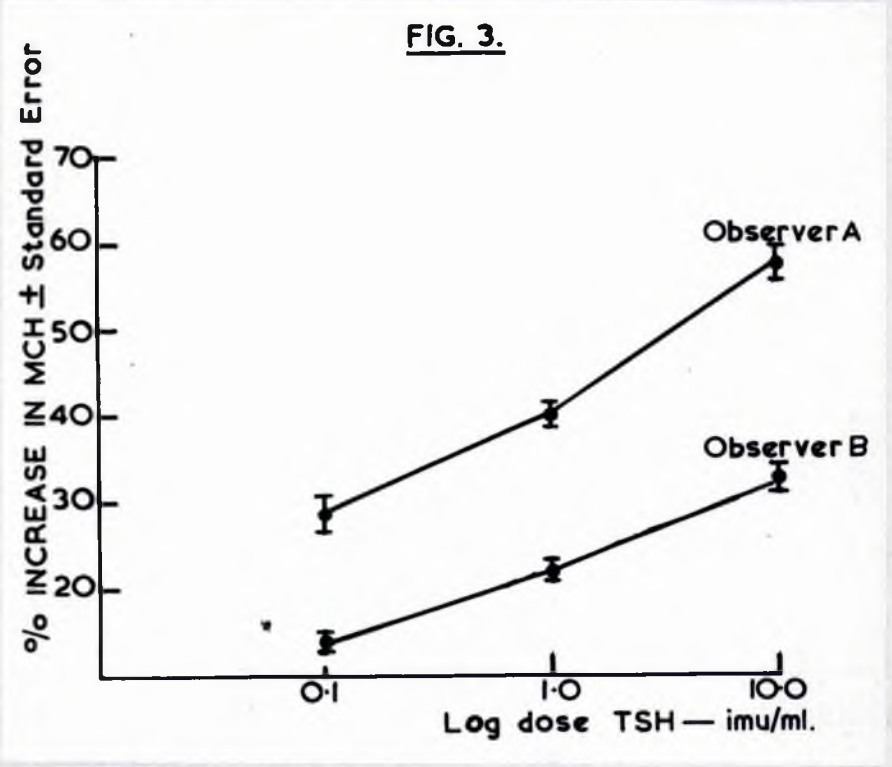

% INCREASE IN MCH ± Standard Error
70
60
50
40
30
20
Observer A
Observer B
0·1
1·0
10·0
Log dose TSH — imu/ml.

TABLE XV

Histometric Assay of International Standard TSH

(Observer B)

	x	x'	x''	x'''
Dosage TSH imu/ml.	10.0	1.0	0.1	Control
Number of animals on which readings were taken	9	7	7	7
MCH of individuals within group (μ)	9.18 9.05 9.13 9.61 9.22 8.57 9.14 9.37 9.07	8.53 8.31 8.62 8.02 8.01 8.41 8.12	7.89 7.91 7.81 7.74 7.88 7.72 7.64	6.93 7.01 6.76 6.90 6.77 6.94 6.96
MCH of group	9.15	8.29	7.79	6.89
S.E. of mean	±0.0933	±0.0933	±0.0382	±0.0362
S.D. of mean	±0.28	±0.246	±0.101	±0.0957
Increase over control	2.26	1.40	0.9	-
% Increase	32.8	22.0	13.5	
± Standard error	±1.02	±1.12	±0.49	

Calculation of results

Statistical calculations were made as previously described:-

(a) Index of precision

$$s_D = 0.1627$$

$$b = 0.68$$

$$\lambda = \frac{b}{s_D} = \underline{0.239}$$

(ii) Test of Significance of difference between the means.

$x - x'$ (10 imu/ml. - 1.0 imu/ml.)

$n = 14$

$s_D = 0.0289$

$t = 29.757$

$p < 0.001$

$x' - x''$ (1.0 imu/ml. - 0.1 imu/ml.)

$n = 12$

$s_D = 0.05905$

$t = 8.4745$

$p < 0.001$

$x'' - x'''$ (0.1 imu/ml. - control)

$n = 12$

$s_D = 0.06155$

$t = 14.622$

$p < 0.001$

The figures obtained by Observer B compare well with those obtained by Observer A. The regression lines (Figure 3) show a good degree of parallelism. These findings indicate that, given sufficient practise, two observers making duplicate sets of readings can achieve a close degree of agreement.

Some method of standardisation should, however, be applied before comparison of results obtained by a series of observers could be considered valid. This could be done by setting up the results obtained by one well-

FIG. 4.

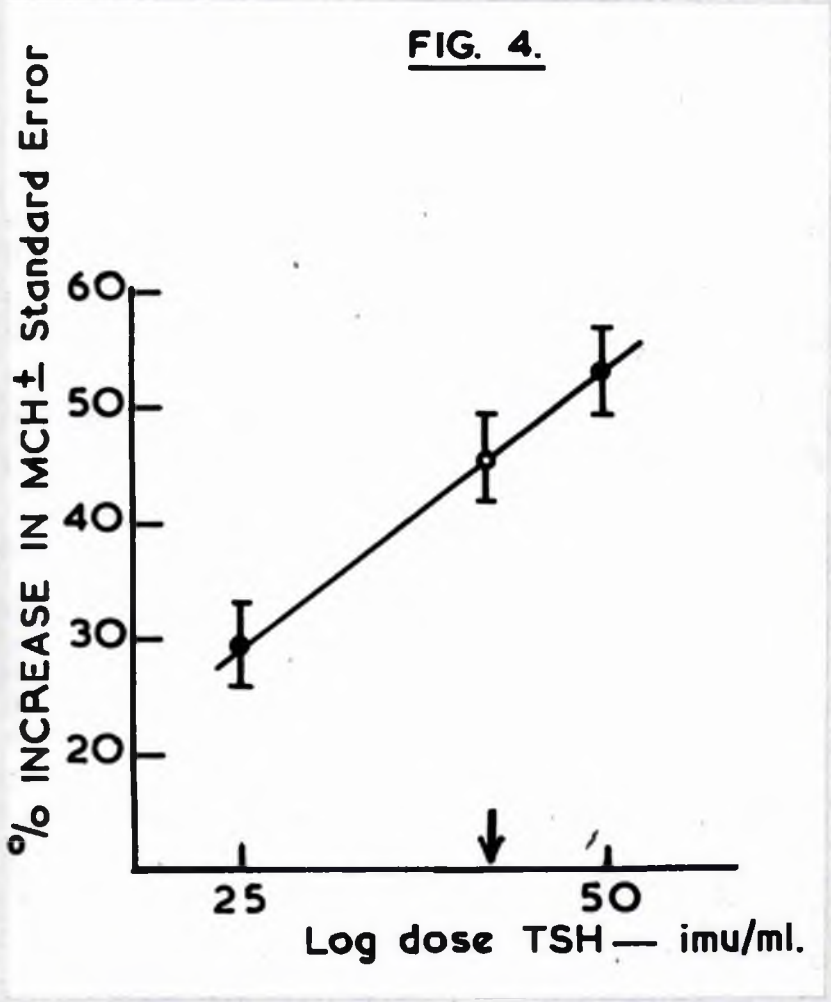


Fig. 4

Assay of Organon pituitary extract

O denotes Organon extract

practised observer as an arbitrary standard. Duplicate readings made by less experienced observers should then be compared with the "standard" readings in terms of the calculated values for standard deviation and slope on a series of estimations.

IV ASSAY OF OX-PITUITARY TSH EXTRACT (ORGANON PREPARATION)

Experimental Routine:-

Number of animals = 24

Starvation period = 8 days

Injection schedule = 0.01 ml/day/4 days

TABLE V

Dosage TSH	Standard 1 50 imu/ml	Standard 2 25 imu/ml	Organon Extract 0.1 mg/ml	Control
Number of animals	6	6	6	6
Mean hind-limb length (mm.)	8.5	8.8	8.4	8.2
MCH of individuals with group (μ)	10.55 10.94 10.37 11.07 11.47 11.10	9.13 9.83 9.61 9.27 8.28 9.32	10.96 9.82 10.37 10.61 10.21 10.41	7.87 6.77 7.98 6.16 6.43 7.58
MCH of group (μ)	10.92	9.24	10.36	7.14
S.E. of mean	± 0.16	±0.13	±0.208	±0.301
S.D. of mean	± 0.392	±0.319	±0.509	±0.737
Increase over control	3.78	2.10	3.22	-
% increase ± standard error	53.0 ± 3.58	29.4 ±3.46	45.1 ±3.74	

It has already been stated that from this type of assay only an approximate value for the potency of the unknown can be obtained. The results (summarised in Table V) are shown in Figure 4. The mean potency of the extract is calculated as 39.4 imu/ml. with a standard error 5.0 imu/ml. Since 0.1 mg. of the Organon preparation was initially dissolved in 1 ml. of isotonic saline the activity in the powder is therefore approximately 0.39 IU/mg. The material was also assayed on two occasions using the method described by Adams & Purves (1955), by K. M. Ferguson at the Getty Marine Laboratory, by which the potency was estimated to be 0.66 IU/mg. with a range of error 0.5 IU/mg - 1.33 IU/mg.

The figure quoted by the manufacturers was arrived at using a modification of the method described by Heyl & Lacquer (1935) and was 1 "Heyl-Lacquer" unit/mg. of powder. Since the "Heyl-Lacquer" unit is said to be equipotent with the Junkmann-Schoeller unit this is equivalent to a potency of 0.1 IU/mg.

This discrepancy draws attention to the question whether a valid comparison can be made between results obtained by methods dependent on end-points requiring different degrees of stimulation and using different test-animals. It is doubtful whether a good degree of parallelism would be expected between results based on:-

(a) Short term physiological effects, as in the method described by Adams & Purves (1955) where ^{131}I discharge is measured 2 hrs. after injection of the test substance, and

(b) A histological method in which the end-point is an arbitrary delimitation of morphological signs of stimulation (Heyl & Lacquer, 1935).

Before making such a comparison, it would be necessary to explore more fully the apparently complex effect of TSH on the thyroid. Further

understanding of the mechanism of action of TSH is required. Direct methods of measuring thyroid stimulation depend on three criteria, (i) hypertrophy of the cells, (ii) uptake of iodine and increase in formation of the hormone, and (iii) increased release of stored hormone. It is not known whether these three demonstrable effects represent different phases in increased thyroid activity or separate aspects of the action of TSH.

V. ASSAY OF FROG PITUITARY EXTRACT

This was a preliminary experiment intended to examine the possibility of applying the assay as a means of assessing the level of pituitary activity in Rana temporaria. It was proposed that it might be used together with other tests to determine the functional status of the pituitary during the annual cycle of activity and hibernation in the frog.

Preparation of Pituitary Extract

The pituitary material for the assay was supplied by Dr. P. G. W. J. van Oordt, Department of Zoology, University of Utrecht.

Acetone dried whole pituitaries from 40 frogs were homogenised with 1.0 ml. of isotonic saline and the suspension left to stand overnight at +2°C. The homogenate was then centrifuged at 3,000 r.p.m. for 20 mins. and the supernatant liquid pipetted off for use in the assay.

Experimental Routine:-

Number of animals = 32

Starvation period = 8 days

Injection schedule = 0.01 ml./day/5 days.

The amount of activity present in the extract was estimated to be 4.07 imu/ml. with a standard error ± 0.45 imu/ml. (Table VI Figure 5). Thus since the extract from 40 glands contained 4.07 imu, that from one gland would contain 0.102 imu. It is probable that this value is too low since some

FIG. 5.

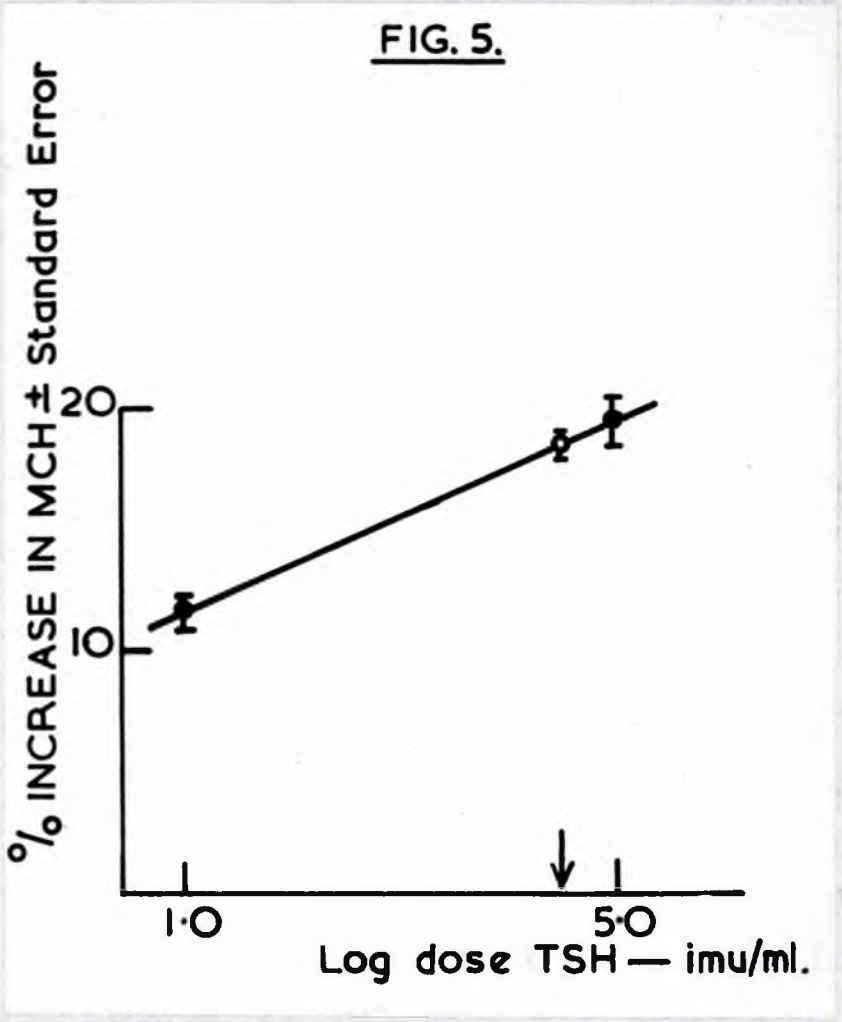


Fig. 5

Assay of Rana temporaria pituitary extract

o denotes Rana material

activity may well have remained in the precipitate after centrifugation. These preliminary findings suggest that the test would prove to be a useful means of assessing pituitary function if frogs are available in sufficient numbers and a more efficient method of extraction is applied. The thyrotrophic activity in the glands could best be expressed on a weight basis.

TABLE VI

Dosage TSH	Standard 1. 5.0 imu/ml.	Standard 2. 1.0 imu/ml.	Rana pituitary extract	Control
Number of animals	6	7	8	8
Mean hind-limb length (mm.)	7.3	7.6	7.7	7.4
MCH of individuals within group (μ).	8.45 8.42 8.38 8.33 8.30 8.85	7.95 8.00 7.97 7.83 7.88 7.82 7.90	8.53 8.27 8.40 8.33 8.72 8.65 8.24 8.02	7.05 6.98 7.14 7.05 6.66 7.28 7.35 7.15
MCH of group (μ)	8.46	7.91	8.39	7.08
S.E. of mean	±0.087	±0.026	±0.027	±0.08
S.D. of mean	±0.195	±0.068	±0.076	±0.226
Increase over control	1.38	0.83	1.31	
% Increase	19.5	11.7	18.5	
± Standard error	±1.03	±0.387	±0.322	

VI. ASSAY OF THYROTROPHIN IN SERUM AND SERUM EXTRACTS

The practicability of the histometric method as applied to clinical studies was investigated using serum obtained from subjects in the majority of whom the TSH-titre was expected to be higher than the euthyroid level. Eight examples of these assays using serum have been described; the results and corresponding dose-response curves are summarised in Table VII - XIV and Figures 6 - 13. In two cases(7 and 8) a simple separation of the serum fractions was attempted. A brief outline of the clinical status of each case is given below:-

1) Mr. A.W. 65 years — Primary myxoedema; symptoms for 1 year.

2) Mrs. M.P. — Post-operative myxoedema; 8 years after operation. No previous treatment.

3) Mrs. M.A. 54 years — Untreated myxoedema.

4) Miss J.B. 50 years — Thyrotoxicosis.

5) John W. 3 weeks. — Thyroid goitre and cretinism.

6) a) Alexander T. 5 years — Large diffuse goitre - not obviously myxoedematous.

b) Miss J.R.B. 25 years — Normal, euthyroid.

7) Mrs. M.P. — Post-operative myxoedema (See case No. 2.)

Whole serum and albumin and globulin fractions tested.

8) Mrs. M.K. — Severe Myxoedema.

Whole serum and 2 globulin fractions tested.

FIG. 6.

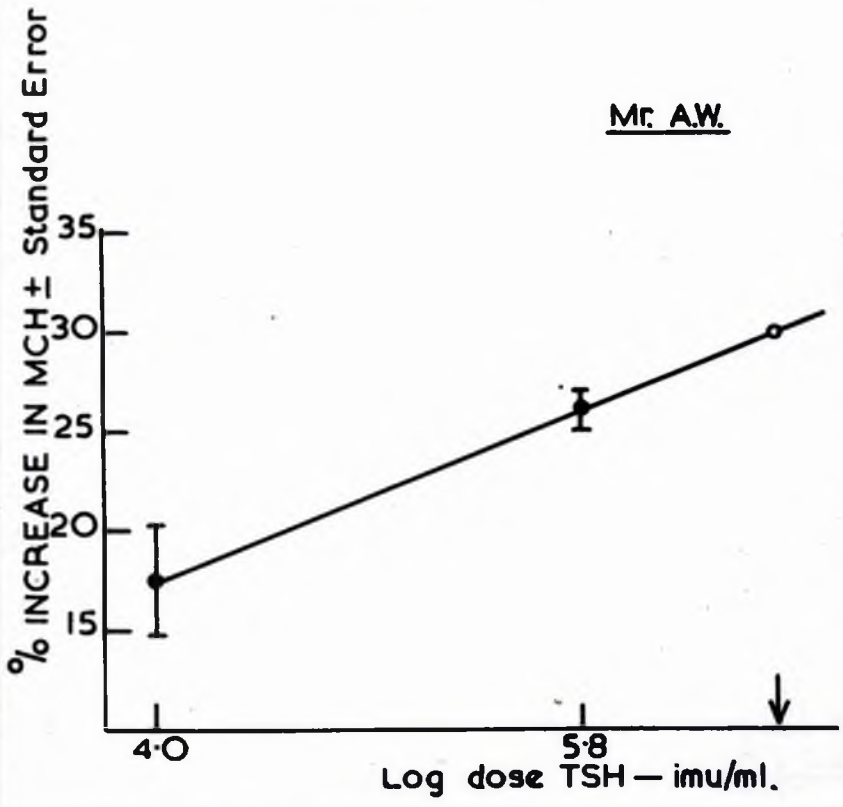


Fig. 6

O denotes serum level

1. Mr. A. W.

Experimental Routine:-

Number of animals = 28

Starvation period = 8 days

Injection schedule = 0.01 ml./day/6 days.

TABLE VII

Dosage TSH	Standard 1. 5.8 imu/ml.	Standard 2. 4.0 imu/ml.	Serum	Control
Number of Animals	7	7	6	7
Mean hind-limb length (mm.)	4.2	4.1	4.2	4.3
TCH of group (μ)	8.59	8.03	8.86	6.83
S.E. of mean	±0.0913	±0.215	±0.115	±0.098
S.D. of mean	±0.242	±0.567	±0.305	±0.240
Increase over control	1.76	1.20	2.03	
% Increase ± Standard error	25.8 ±1.07	17.6 ±2.68	29.8 ±1.30	
Estimated TSH-titre			>5.0 imu/ml. approx. 6.9 imu/ml.	

FIG. 7.

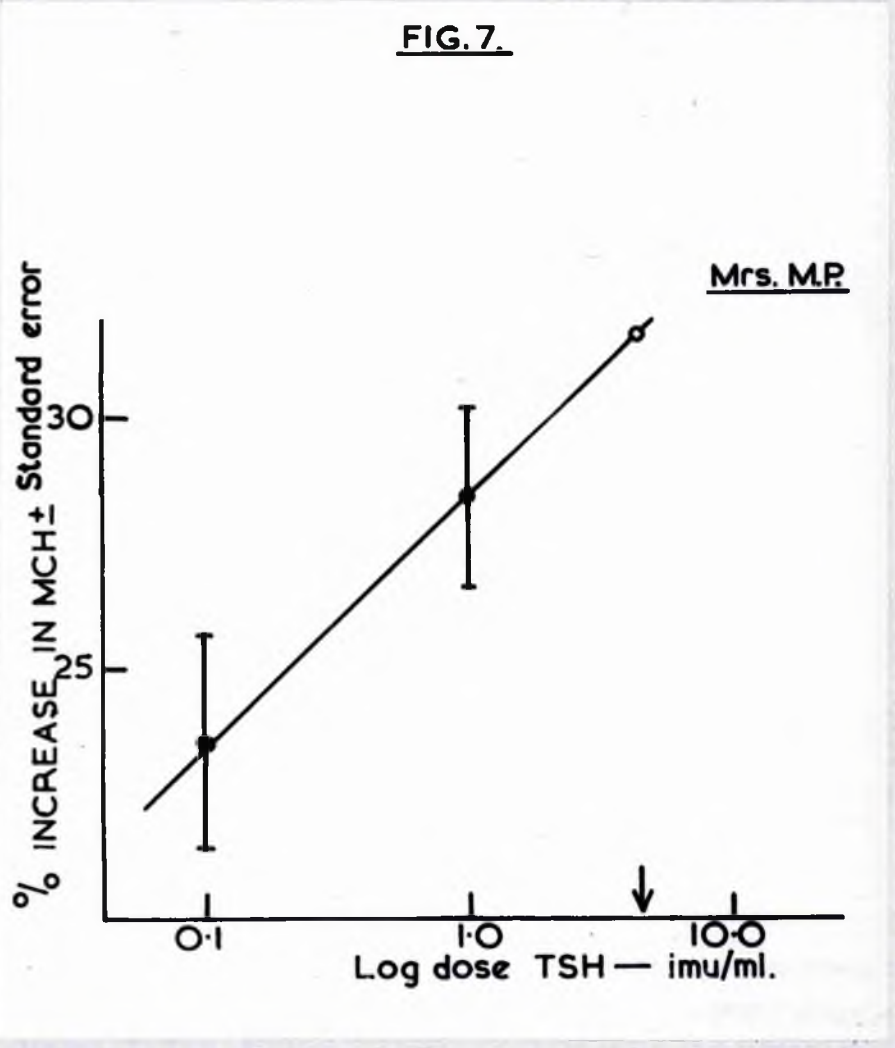


Fig. 7

O denotes serum level

2. Mrs. M.P.

Experimental Routine:-

Number of animals = 28

Starvation period = 8 days

Injection schedule = 0.02 ml/day/5days.

T A B L E VIII

Dosage TSH.	Standard 1. 1.0 imu/ml.	Standard 2. 0.1 imu/ml.	Serum	Control
Number of animals	6	7	7	7
Mean hind-limb length (mm.)	3.6	3.8	4.2	2.9
MCH of group (μ)	8.76	8.42	8.97	6.82
S.E. of mean	±0.16	±0.18	±0.14	±0.09
S.D. of mean	±0.394	±0.475	±0.369	±0.237
Increase over control	1.94	1.60	2.15	
% Increase	28.4	23.5	31.6	
± Standard error	±1.83	±2.14	±1.56	
Estimated TSH-titre			> 1.0 imu/ml. approx. 4.4 imu/ml.	

FIG. 8.

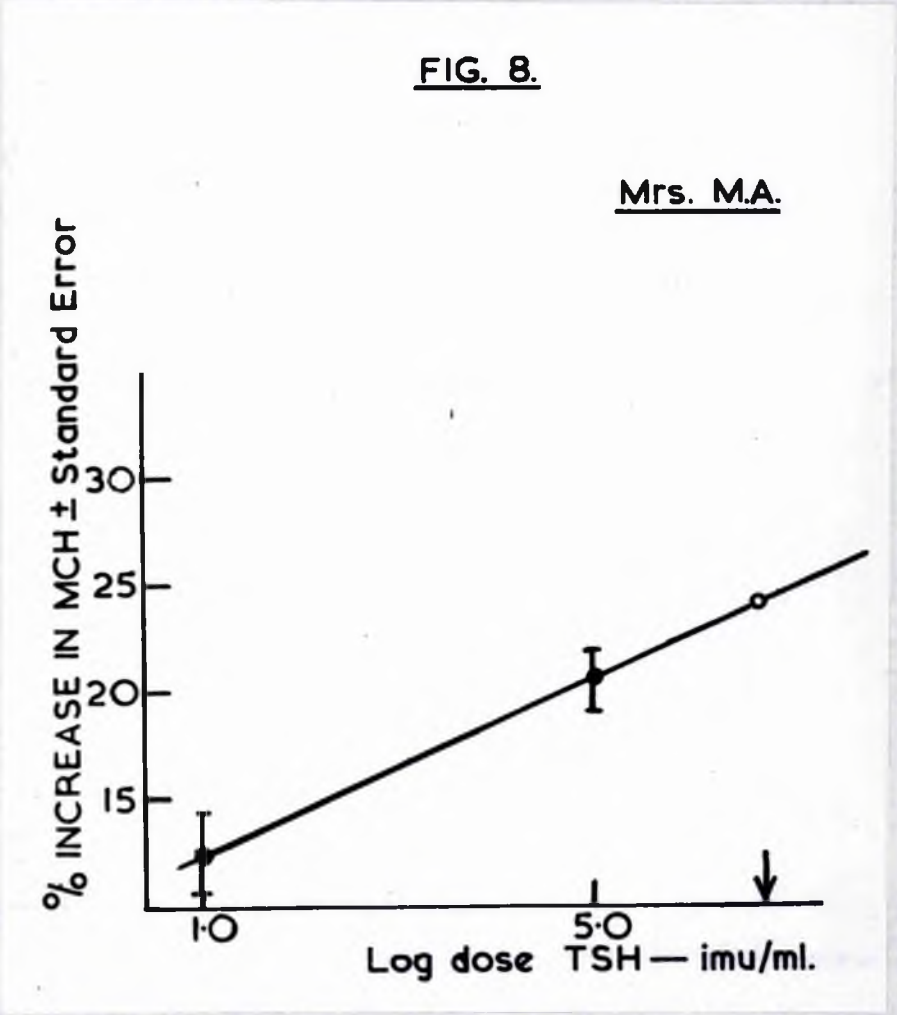


Fig. 8

O denotes serum level

3. Mrs. M.A.

Experimental Routine:-

Number of animals = 24

Starvation period = 8 days.

Injection schedule = 0.01 ml./day/6 days.

TABLE IX

Dosage TSH	Standard 1. 5 imu/ml.	Standard 2. 1 imu/ml.	Serum	Control
Number of animals	6	5	5	5
Mean hind-limb length (mm.)	4.5	4.6	4.3	3.8
MCH of group (μ)	8.47	7.91	8.73	7.03
S.E. of mean	±0.114	±0.149	±0.093	±0.094
S.D. of mean	±0.278	±0.333	±0.208	±0.212
Increase over control	1.44	0.88	1.70	
% Increase ± Standard error	20.5 ±1.345	12.5 ±1.88	24.2 ±1.065	
Estimated TSH-titre			> 5.0 imu/ml.	

FIG. 9.

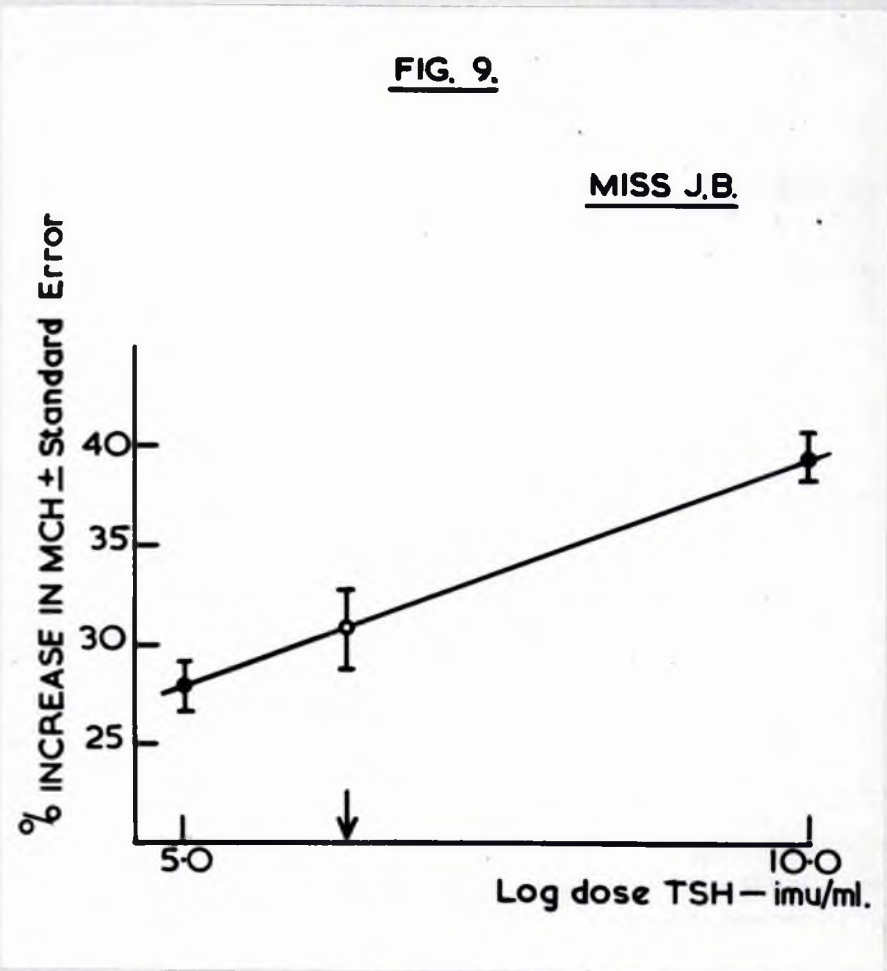


Fig. 9

O denotes serum level

4. Miss J.B.

Experimental Routine:-

Number of animals = 28

Starvation period = 8 days

Injection schedule = 0.01 ml./day/5 days.

TABLE X

Dosage TSH	Standard 1 10 imu/ml.	Standard 2. 5 imu/ml.	Serum	Control
Number of animals	7	7	6	3
Mean hind-limb length (mm.)	4.1	4.1	5.9	4.9
MCH of group (μ)	8.90	8.16	8.34	6.38
S.E. of mean	±0.106	±0.097	±0.178	±0.047
S.D. of mean	±0.281	±0.259	±0.437	±0.081
Increase over control	2.52	1.78	1.96	
% Increase	39.5	27.9	30.8	
± Standard error	±1.195	±1.19	±2.14	
Estimated TSH-titre			5.98 imu/ml. (5.25 - 6.76) imu/ml.	

FIG. 10.

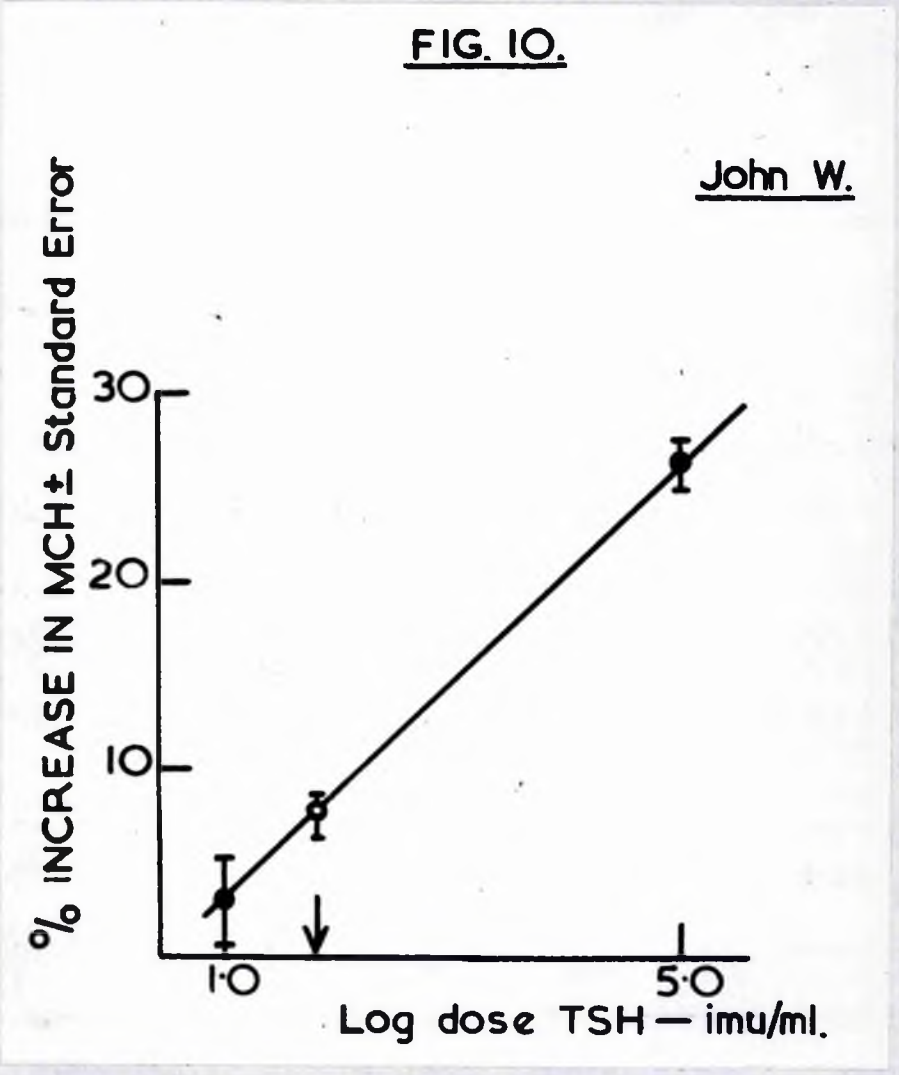


Fig. 10

o denotes serum level

5. J.W.

Experimental Routine:-

Number of animals = 20

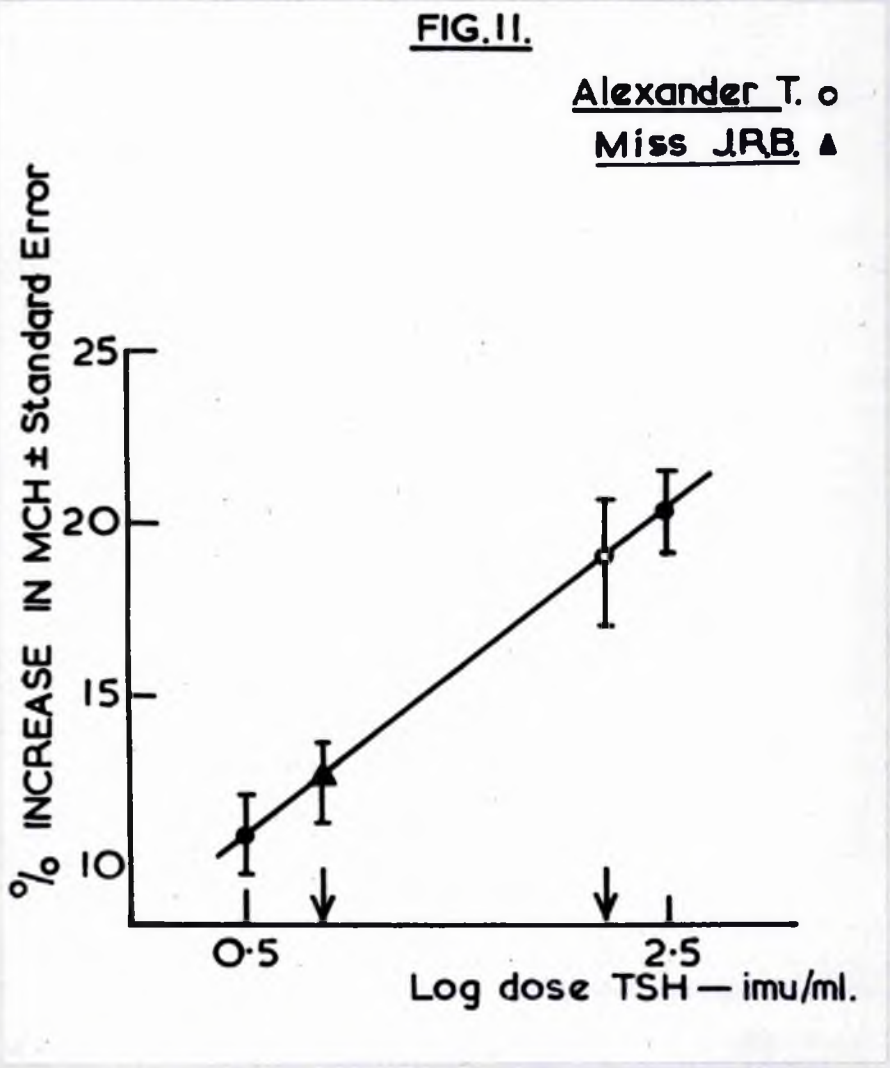
Starvation period = 8 days

Injection schedule = 0.02 ml/day/6 days.

TABLE XI

Dosage TSH	Standard 1. 5.0 imu/ml.	Standard 2. 1.0 imu/ml.	Serum	Control
Number of animals	5	3	3	4
Mean hind-limb length (mm.)	4.6	4.9	4.0	4.8
MCH of group (μ)	9.28	7.62	7.88	7.34
S.E. of mean	±0.128	±0.189	±0.074	±0.203
S.D. of mean	±0.287	±0.328	±0.128	±0.406
Increase over control	1.94	0.28	0.54	
% Increase ± Standard error	26.4 ±1.38	3.82 ±2.48	7.37 ±0.94	
Estimated TSH-titre			Approx. 1.38 imu/ml. (1.25 - 1.50) imu/ml.	

FIG. II.


Alexander T. o
Miss J.R.B. ▲
% INCREASE IN MCH ± Standard Error
25
20
15
10
0·5
2·5
Log dose TSH — imu/ml.

5. (a) Alexander T. (b) Miss J.R.B.

Experimental Routine:-

Number of animals = 35

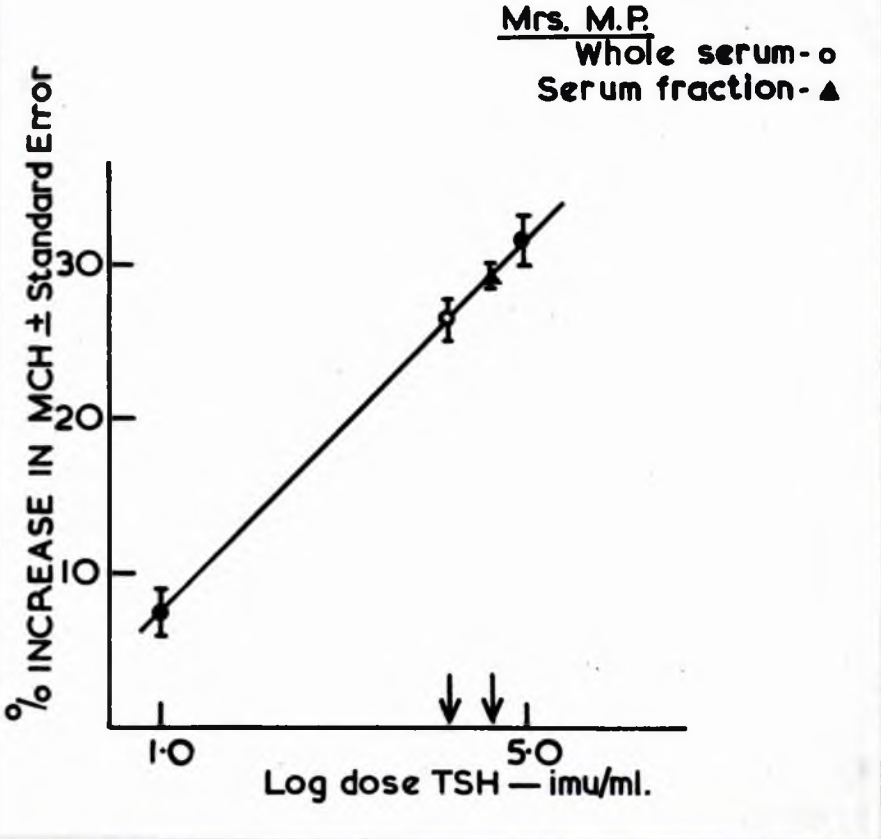
Starvation period = 8 days

Injection schedule = 0.01 ml./day/6 days.

TABLE XII

Dosage TSH	Standard 1. 2.5 imu/ml.	Standard 2. 0.5 imu/ml.	Serum (a)	Serum (b)	Control
Number of animals	5	5	6	5	7
Mean hind-limb length (mm.)	3.6	4.46	4.03	5.8	3.7
MCH of group (μ)	8.06	7.43	7.96	7.55	6.70
S.E. of mean	±0.109	±0.096	±0.159	±0.064	±0.062
S.D. of mean	±0.246	±0.203	±0.398	±0.142	±0.163
Increase over control	1.36	0.73	1.26	0.85	
% Increase	20.3	10.9	18.8	12.7	
± Standard error	±1.35	±1.21	±2.0	±1.13	
Estimated TSH-titre			approx. 1.95 imu/ml. (1.4-2.6 imu/ml.)	approx. 0.66 imu/ml. (0.55-0.8 imu/ml.)	

FIG. 12.


Mrs. M.P.
Whole serum - o
Serum fraction - ▲
% INCREASE IN MCH ± Standard Error
30
20
10
1·0
5·0
Log dose TSH — imu/ml.

7. Mrs. M.P.

Experimental Routine:-

Number of animals = 30

Starvation period = 8 days

Injection schedule = 0.02 ml./day/4 days.

TABLE XIII

Dosage TSH	Standard 1. 5.0 imu/ml.	Standard 2. 1.0 imu/ml.	Whole Serum	Globulin Fraction	Albumin Fraction	Control
Number of Animals	5	4	3	4	-	3
Mean Hind-limb length (mm.)	5.4	5.6	5.5	4.7	-	6.0
MCH of group (μ)	8.57	6.99	8.22	8.40	-	6.51
S.E. of mean	±0.123	±0.116	±0.103	±0.061	-	±0.111
S.D. of mean	±0.275	±0.233	±0.178	±0.121	-	±0.192
Increase over control	2.06	0.48	1.71	1.89	-	-
% Increase	31.6	7.36	26.2	29.1	-	-
± Standard error	±1.43	±1.66	±1.25	±0.715	-	-
Estimated TSH-titre			3.5 imu/ml. (3.2-3.9 imu/ml.	4.2 imu/ml. (3.9 - 4.5 imu/ml.		

Preparation of serum fractions.

A second assay was performed on the serum of Mrs. M.P. (Case 2.) in which the activity in untreated serum was compared with that in the globulin and albumin fractions. The globulin and albumin fractions were separated by a simple fractionation procedure. 1 ml. of whole serum was diluted with 1 ml. of distilled water. 2 ml. of saturated ammonium sulphate was then added slowly over a period of 10 minutes. The mixture was stirred for 2 hours, left to stand at +2°C. overnight then centrifuged.

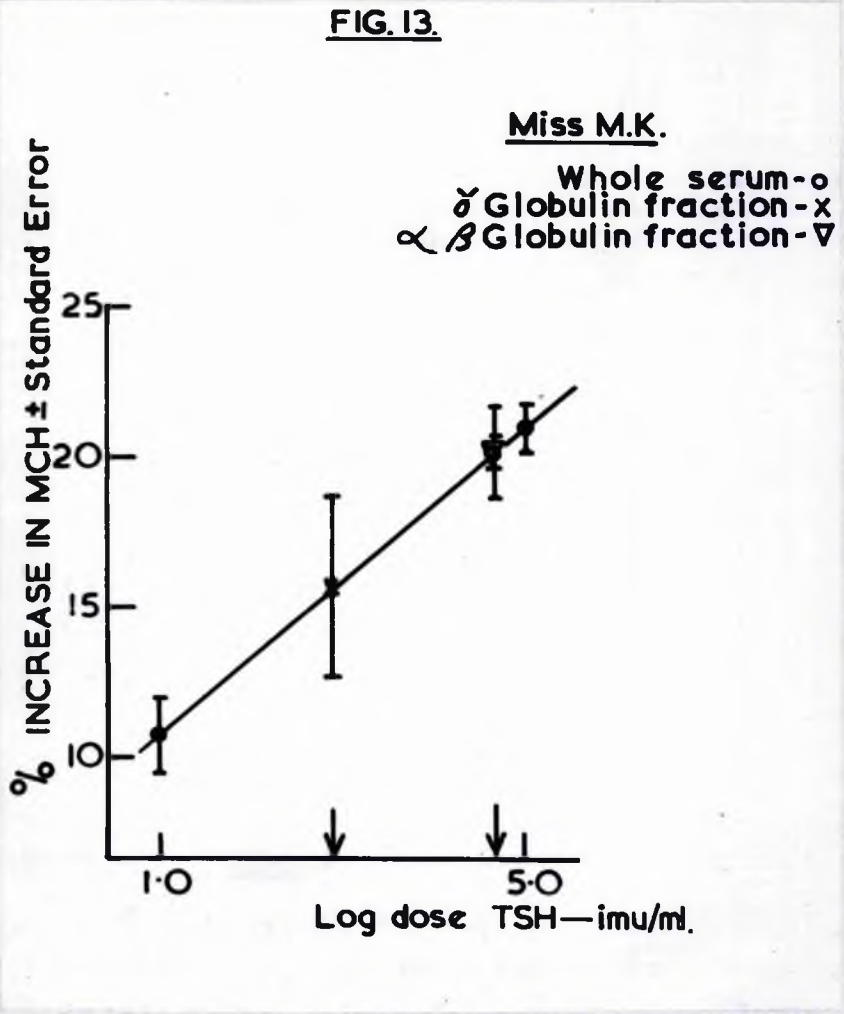
(1.) The globulin precipitate obtained was mixed with 2 ml. of 50% ammonium sulphate solution, stirred for 1 hr. and centrifuged.

(2.) The precipitate was dissolved in 5 ml. of water and dialysed against distilled water to free the globulin fraction of ammonium sulphate and finally freeze-dried.

The supernatant liquid from (1) above was dialysed until free from ammonium sulphate and the albumin fraction obtained was freeze dried. For injection into the test animals the globulin fraction was made up in 0.6 ml. of isotonic saline and the albumin fraction in 0.9 ml. Both fractions were rather insoluble, the albumin more so than the globulin; it formed a thick white suspension. The albumin fraction proved to be severely toxic and all the animals in this group were dead within 15 minutes of the first injection.

Unfortunately, the size of the test groups is too small to obtain exact figures for the potency of the solutions injected but the globulin fraction is shown to contain a higher TSH-titre than the whole serum in the ratio 1.2 ; 1.0.

FIG. 13.


Miss M.K.
Whole serum-o
δ Globulin fraction-x
α β Globulin fraction-∇
% INCREASE IN MCH ± Standard Error
25
20
15
10
1·0
5·0
Log dose TSH—imu/ml.

8. Miss M.K.

Experimental Routine:-

Number of animals = 42

Starvation period = 8 days

Injection schedule = 0.01 ml/day/6days.

TABLE XIV

Dosage TSH	Standard 1. 5.0 imu/ml.	Standard 2. 1.0 imu/ml.	Whole Serum	γ-Globulin Fraction	α-β-Globulin Fraction	Control
Number of animals	6	6	7	5	7	7
Mean hind-limb length (mm.)	2.5	2.6	2.3	3.1	2.9	2.8
MCH of group (μ)	8.54	7.82	8.49	8.16	8.49	7.06
S.D. of mean	±0.163	±0.228	±0.403	±0.549	±0.159	±0.211
S.E. of mean	±0.067	±0.093	±0.153	±0.245	±0.060	±0.079
Increase over control	1.48	0.76	1.43	1.10	1.43	
% Increase	21.0	10.75	20.2	15.6	20.2	
± Standard error	±0.703	±1.19	±1.805	±3.0	±0.71	
Estimated TSH-titre			4.36 imu/ml. (3.46 - 5.62) imu/ml.	2.14 imu/ml. (1.35 -3.46 imu/ml.)	4.36 imu/ml. (4.07 -4.78 imu/ml.)	

Preparation of serum fractions.

The fractionation procedure followed was similar to that described for Case 7. In view of the highly toxic effect observed on injection of the albumin fraction, this was discarded. Serum globulins were further separated into a γ-globulin fraction and α-β-globulin fraction.

1 ml. of serum was diluted with 1 ml. of distilled water and 2 ml. of saturated ammonium sulphate was added slowly over half an hour with stirring. The mixture was refrigerated for 2 hours then centifruged. The globulin precipitate was dissolved in 0.4 ml. of phosphate buffer at pH 8 and 60 mg. of sodium sulphate added. This was stirred for 1 hour and centifruged. The γ-globulin precipitate was dissolved in saline and dialysed. The α-β-globulin fraction in the supernatant was also dialysed and both fractions were freeze dried. The two fractions were made up in 0.5 ml. isotonic saline for injection. The α-β-globulin fraction was found to be much more soluble than the γ-globulin fraction.

Summary of Results

The results obtained in the preceding series of assays are summarised below. With the limited material available it was only possible to obtain an approximate value for the activity present in the serum tested. Where the response obtained to injection of serum fell outside the points on the standard dose-response curve the potency of the "unknown" was expressed as "greater than" or "less than" the dose-level of the standard concerned. Table XV contains a summary of the findings detailed in Tables VII - XIV.

T A B L E XV

	Untreated Serum	Serum fractions	
1) Mr. A.W.	>5.0 imu/ml.		
2) Mrs. M.P.	>1.0 imu/ml.		
3) Mrs. M.A.	>5.0 imu/ml.		
4) Miss J.B.	approx. 5.98 imu/ml.		
5) John W.	approx. 1.38 imu/ml.		
6) (a) Alexander T.	approx. 1.95 imu/ml.		
(b) Miss J.R.B.	approx. 0.66 imu/ml.		
7) Mrs. M.P.	approx. 3.5 imu/ml.	Total Globulin approx. 4.2 imu/ml.	Albumin - Toxic
8) Miss M.K.	approx. 4.36 imu/ml.	γ-Globulin approx. 2.14 imu/ml.	α-β-Globulin approx. 4.36 imu/ml.

Discussion.

The "semiquantitative nature of assays performed using small test-groups has already been discussed (page 35). There are, however, several important facts arising from these attempts to apply the method to measurement of TSH in human serum.

In cases 1-3, the TSH-titre was found to be high, ranging from >1.0 to>5.0 imu/ml. This is in fair agreement with the findings of D'Angelo et al. (1950) who quote a range of zero - 3.75 imu/ml. for the level of circulating TSH in hypothyroid subjects. It is of interest to note that, in case 2, where the condition is post-operative in origin and there is a clinical history of symptoms increasing in severity over a period of eight years, the TSH-titre appears to be somewhat lower than in cases 1 and 3 where the hypothyroid condition is of comparatively recent origin. Similar observations have been reported by Emmerson & Cutting, 1938, De Robertis, 1948 and Gilliland & Strudwick, 1956. These findings have been attributed to the gradual failure of pituitary secretion of TSH in cases of long-standing myxoedema.

A high TSH-titre was found in Case 4, the value being approximately 6 imu/ml. of serum. The range of concentration of TSH in thyrotoxicosis quoted by D'Angelo et al. (1950) is zero - 1.5 imu/ml. The value obtained in this assay is therefore rather higher than would be expected from previous findings in hypothyroid subjects.

Cases 5 and 6a are of interest in that both children were related to the families in which a study of inherited goitre and cretiniam was made by Hutchison and McGirr (Hutchison, 1956, Hutchison & McGirr, 1954, 1956). A measurable amount of TSH was demonstrated in both cases. Little work has been done on thyrotrophin levels in this age group, but Di George, D'Angelo & Paschkis (1957) estimated the TSH level in a nine day old

euthyroid boy to be equivalent to 0.4 µg. Parke-Davis thyrotrophin preparation per ml., i.e. 0.8 imu/ml. (1 µg. of Parke-Davis preparation = 2 imu U.S.P. reference standard). In a cretin of 11 years of age, Di George et al. (1957) found no detectable TSH prior to treatment and later a high TSH-titre after thyroid therapy.

With reference to this apparent recovery of TSH secretion these workers comment that both duration and severity of the condition may be contributary factors in determining the interval before complete failure of TSH secretion occurs. A survey of thyrotrophin levels in the new born infant and young child would provide useful information, not only on the normal euthyroid status, but also on the equivocal problem of placental transmission of maternal thyrotrophin. The latter problem has been discussed by several groups of workers, Grumbach & Werner, 1956, Skelton & Gans, 1955, and Lewis & McGregor, 1957).

In Case 6b a high positive response for TSH was obtained to injection of a euthyroid serum. A large series of euthyroid man and woman would have to be studied before the range of euthyroid levels is established. Bottari (1959) has demonstrated that in the normal, healthy woman, during reproductive life, TSH levels are generally higher than in men and in post-menopausal women.

A number of attempts have been made to develop a fractionation technique whereby a concentrated extract containing TSH could be prepared from serum (Lamaijer, Kassenaar & Querido, 1955, Lamaijer, 1956, Postel, 1956, McKenzie, 1958). The fractionation method employed in Cases 7 and 8 was rather crude; a positive response was obtained with all the globulin fractions tested. Solubility is an important factor where small amounts of material are available. The globulin fractions tested were found to be rather

insoluble and were injected as a thick white suspension.

Another problem arises in this connection. The volume injected was relatively large in proportion to the size of the test-animal. Injection of a concentrated protein fluid of this description may well elicit a non-specific response due to purely nutritive effects in the "stasis" tadpole. This possible effect could be investigated by injection of a serum known to contain no TSH.

Future work in the field of TSH assay clearly demands a detailed examination of possible fractionation methods. The major obstacle to estimation of TSH in body fluids is lack of sensitivity in all but a few of the available methods. Preparation of a concentrated serum extract containing TSH by precipitation with alcohol and acetone, electrophoresis or chromatography could be usefully combined with one of the simpler techniques of lesser sensitivity.

In the assays carried out, two dose-levels only of the Standard preparation were used because of the limited number of animals available; the values obtained for the TSH concentration in the sera tested were therefore approximate. An accurate determination of the TSH-titre can only be obtained when a 3-point dose-response curve is constructed for the Standard. The results obtained in this group of assays performed on sera from patients exhibiting various thyroid disorders, however, indicate that the histometric method can be applied clinically to demonstrate changes in the level of circulating TSH provided that larvae of a suitable size are available in sufficient numbers and that the time taken to complete the estimation is not of major importance.

VII. MEASUREMENT OF ACINAR CELL-HEIGHT BY A PROJECTION METHOD.

In the preceding sections it has been demonstrated that an acceptable degree of precision and sensitivity in assaying TSH by increase in acinar cell-height can be achieved only by using relatively large groups of animals at each dose-level. The number of assays which can be performed in a given time on the required scale is dependent upon two factors:-

1. The number of suitable animals available.
2. The time required for preparation of the sections and measurement of cell-heights.

Some previous attempts to short-cut the time-consuming procedure of making histometric measurements have already been discussed (pages 6 and 28). A modification of the method of linear measurement, using an enlarged projected image of the thyroid section, was examined with three objects in view:-

1. To speed up the process of making the measurements and at the same time to reduce the strain imposed upon the operator.
2. To reduce the subjective element involved.
3. To determine whether variability in the results is reduced if, as a result of increased ease of measurement, the number of readings taken is greatly increased.

PROJECTION APPARATUS

The apparatus was constructed to project an image of the thyroid section vertically downwards onto a sheet of white card. The source of illumination was a powerful motor-cycle head-lamp arranged so that the beam of light was directed down a black cardboard tube onto the condenser of an inverted microscope. Both light-source and microscope were attached to a

stable vertical support on which they could be racked up or down independently so as to give optimal lighting. The distance between the eyepiece of the microscope and the "screen" (approx. 2½ ft.) was such that maximum magnification with good definition was achieved in the resulting image. It was found that definition was lost with an image of diameter greater than 1 ft. The preparation was placed, cover-slip down, on the upper side of the microscope stage. To exclude light a black-out curtain was hung from the apparatus; this completely enclosed the table on to which the image was projected.

METHOD OF MEASUREMENT

Acinar cell-height, i.e. the distance between the basement membrane and the inner wall of the cell, was measured by placing the edge of a piece of stiff paper across the centre of the cell and marking its height on the edge of the paper. The next cell was then measured by placing the paper so that the second stroke made in measuring the previous cell corresponded to the external border. This procedure was repeated for the required number of cells so that each individual cell-height was represented between two strokes on the paper. The mean cell-height was derived by dividing the figure obtained for the total length of the strip of paper on which the measurements were recorded by the number of cells measured. Using a sharp pencil, the thickness of the marking strokes corresponded almost exactly with the thickness of the cell-membrane as it appeared at the magnification employed. The apparatus was calibrated using the same slide as was employed to calibrate the eye-piece micrometer. It was found that 1 mm. corresponded to 1.74μ.

By this method of recording cell-heights, many more cells can be measured in a given time than by use of the eye-piece micrometer and with much less strain. For purposes of comparison with the histometric method, readings were made by the projection method on the sections used in obtaining the

results recorded in Table III (page 39). Two sets of readings were taken; in the first, large numbers of cells were measured on small groups of animals (Table XVIa) and in the second, a smaller number of cells were measured in large groups of animals. (Table XVIb)

TABLE XVIa

Dosage TSH ium/ml.	10.0	1.0	0.1	Control
Number of animals	5	5	5	5
Average number of cells measured	222	266	189	199
MCH of group (μ)	7.46	5.71	5.12	4.35
S.E. of mean	±0.21	±0.21	±0.124	±0.089
S.D. of mean	±0.47	±0.47	±0.26	±0.20
Increase over control	3.11	1.36	0.97	-
% increase	71.5	31.12	22.16	-

FIG. 14.

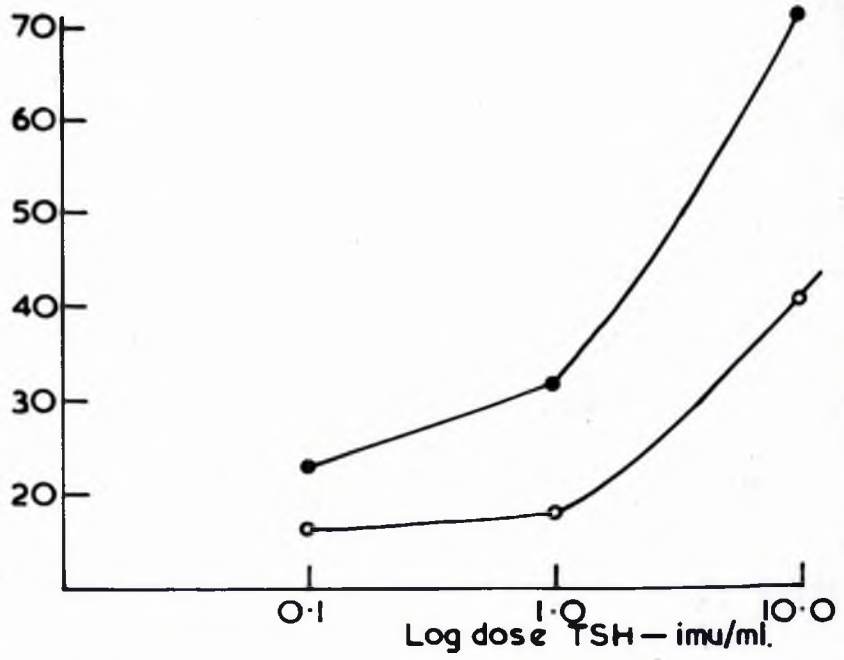


Fig. 14

Dose response curve constructed by the projection method

● measuring >100 cells: 5 animals / dose level

○ measuring 40 cells: 10 animals / dose level

TABLE XVIb

Dosage TSH imu/ml.	10.0	1.0	0.1	Control
Number of animals	10	10	10	10
Number of cells measured	40	40	40	40
MCH of group (μ)	8.09	6.75	6.68	5.75
S.E. of mean	$\pm$0.141	$\pm$0.083	$\pm$0.019	$\pm$0.109
S.D. of mean	$\pm$0.447	$\pm$0.264	$\pm$0.061	$\pm$0.346
Increase over control	2.34	1.00	0.93	-
% Increase	40.7	17.4	16.2	-

Discussion

These results are not entirely conclusive. Two possible causes are suggested to account for the discrepancy in the values for actual cell-height obtained in the two sets of readings:-

1. Insufficient practice in the technique.
2. Loss of definition together with distortion of the image at the edges of the field as a result of the high order of magnification employed.

Both of these sources of error could be reduced, if not eliminated.

An explanation of apparent loss of linearity in the dose-response curve is not immediately apparent although the second source of error mentioned above may be a contributary factor. Error arising from distortion

could be eliminated by restricting the area in which measurements are made to the centre of the field where the image is not affected by the curvature of the lens. It is of interest to note that the shape of the curve obtained is the same with both smaller and larger groups of animals. (Figure 14).

Both the time required to make a given number of measurements and strain on the operator are greatly reduced by this technique as compared with use of the ocular micrometer. There is not the same need for deliberate selection of the cells to be measured and the number of measurements which can be made is greatly increased. The projection method is therefore less subjective than the technique employing the ocular micrometer.

In this preliminary study, all the possibilities of the projection method have not been fully explored. The method of recording the cell-heights is rapid and reliable. Reduction of error depends upon achieving perfect definition and taking measurements only in the centre of the field where the least distortion of the image occurs. The regression line does not change appreciably according to the number of cells measured in each animal and variability between animals has been shown to be greater than variability within one individual. It therefore appears that a projection technique, which would enable the observer to make readings on a greater number of animals than by the micrometer method, would be advantageous in obtaining results of improved reliability and objectivity.

VIII. SUMMARY AND CONCLUSIONS

The findings recorded in this section demonstrate that, using sufficiently large groups of animals, TSH can be detected at a concentrati of 0.1 imu/ml. by the histometric method. Analysis of the results by the accepted statistical methods showed that, when the assay is performed on test-groups of ten or more animals, the reliability of the method is reasonably satisfactory; the index of precision (λ) was found to be 0.38. The same order of sensitivity and reliability is not achieved when test-groups of fewer than ten animals are employed. Where this is the case, the results obtained must be regarded as "semi-quantitative". It is possible, however, by this means, to demonstrate the presence of TSH in serum from patients suffering from myxoedema. An accurate estimation of the TSH-titre in blood could be obtained using larger groups of animals.

In conducting the series of assays performed on serum, it was necessary to reduce the size of the test-groups because of the difficulti in obtaining large numbers of tadpoles suitable for use with the technica assistance available. Such small scale assays also reduce the time required to complete the histometric measurements. A truly quantitative estimation of the serum TSH level must be made for the assay to have meaningful application in connection with clinical investigations. Ther is no reason why this can not be achieved given sufficient technical assistance to maintain a population of larvae of a size to provide large numbers of animals suitable for assay work.

It becomes apparent, from this trial of the method, that the or of sensitivity and precision are such that it may be regarded as a useful research tool. The major obstacle, so far as concerns its clinical application, lies in the time and labour involved in completing an

estimation on the scale necessary to obtain satisfactory results according to the accepted reliability criteria.

A more detailed examination of the projection method should result in the development of a technique by which the rapidity and objectivity of the method would be increased. Use of such a procedure, would greatly reduce the time required to complete an estimation; the assay could then be applied in clinical investigations.

PART III

FACTORS INFLUENCING ^{131}I - ACCUMULATION

PART III

FACTORS INFLUENCING ^{131}I - ACCUMULATION

I. INTRODUCTION

The use of radioactive isotopes in all fields of biological research, and in bioassay in particular, has become increasingly widespread in the past two decades. Radiometric methods have the advantage of being completely objective and, in general, the time required to complete an assay is short. As a rule, the techniques involved are sufficiently simple to be adapted to routine use on a large scale, under circumstances where other more complicated methods have proved to be unsatisfactory. Since the histometric method must be considered to have a limited application in investigations where the time required to complete an assay is of major importance, the possibility of developing a less time-consuming method, dependent on radiometric criteria, was explored.

The pattern of iodine uptake in the course of metamorphosis and in the young toad has previously been described in *Xenopus* using ^{131}I (Dodd, 1955, Saxen *et al.* 1957a & 1957b) and in *Rana glandulosa* larvae during normal and accelerated metamorphosis using both ^{131}I and ^{32}P (D'Angelo, 1956).

Autoradiographic techniques were employed by Dent & Hunt (1955) to study changes in thyroidal activity during metamorphosis in *Hyla*, *Bufo* and *Rana* and by Gorbman & Evans (1941), who demonstrated the early initiation of iodine accumulation in the developing thyroid in the larvae of *Hyla*. The turnover of ^{131}I in normal, thiouracil-treated and TSH-treated larvae of *Rana pipiens* was studied by Money, Lucas & Rawson (1955).

Before investigating possible methods of estimating TSH by uptake or discharge of ^{131}I, it was necessary to examine the factors influencing ^{131}I accumulation in the tadpole thyroid. Accordingly, a study was made of changes

in iodine uptake in the normally metamorphosing larva and young toad, and of the influence of diet and temperature on iodine uptake. The latter investigations were made with a view to the application of control of diet and temperature in the development of an assay technique and therefore were of a preliminary nature. Possible assay techniques, dependent on estimation of ^{131}I - uptake and ^{131}I - discharge were then examined and are described in Parts V and VI.

Plate XII.

Thyroidectomy technique in the *Necturus* larva; the pigmented glands are shown, illuminated from below, lying just anterior to the heart.

Magnification x 120

II. METHODS.

(a) Thyroidectomy

^{131}I-uptake investigations were based on the estimation of the amount of radioactive iodine accumulated by the thyroid glands after the tadpoles had been immersed for a period in a dilute solution of carrier-free ^{131}I in tap-water. Reference has been made to the technique of thyroidectomy in the Xenopus tadpole by Dodd & Landgrebe (1953) and to the procedure for estimation of the radioactivity present in the isolated glands by Dodd (1955) and Brown & Dodd (1956).

In preparation for removal of the thyroid glands, after immersion in ^{131}I for the required period, the tadpoles were washed for 30 minutes in ^{131}I-free water. They were then anaesthetised in 1% urethane and placed ventral side up on damp cotton wool under a binocular dissecting microscope. The area in which the thyroids lie was illuminated from below by placing in the mouth of the tadpole a pointed glass rod concentrating light from a shielded lamp (Plate III). The thyroid glands, located in the bright spot of light thus produced, are enclosed in a tough elastic capsule and were dissected out free from any extraneous tissue by use of tungsten needles and Dumont forceps. They were then transferred to a small drop of water in the centre of an aluminium planchette and evaporated to dryness over a lamp.

The two thyroid lobes are entirely separate in the tadpole and are generally ellipsoid in shape. Some are heavily pigmented and others completely devoid of pigment so that, under high magnification, the individual follicles are clearly distinguishable. In many larvae small subsidiary masses of thyroid tissue were observed, consisting of one or two follicles lying in a subdermal position. Although it was not possible to remove these subsidiary follicles a record was kept of their occurrence and of the size, shape and

degree of pigmentation of the glands throughout all experiments involving thyroidectomy.

(b) Radiometric Methods.

The amount of radioactivity present in the excised thyroids and in liquid samples was determined using standard Panax counting equipment. The majority of radiometric studies were carried out by immersing the test-animals in a dilute solution of ^{131}I. For this purpose ^{131}I solutions were prepared by addition of carrier-free ^{131}I to the total volume of tap-water required. This was then distributed between the jars in which the tadpoles were exposed to ^{131}I, in groups of 10 per 1000 ml. of ^{131}I solution. In all immersion experiments a "blank" jar of ^{131}I solution containing no animals was prepared as a means of checking the normal course of radioactive decay during the experimental period. The amount of ^{131}I taken up by the tadpoles could thus be determined by comparing the activity in the "blank" jar with that in the jar in which the test-animals had been immersed. The radioactivity in the water was determined by counting 9.0 ml. aliquots of the ^{131}I solution in a Veall liquid counter.

The activity present in the dry thyroids was estimated using an end-window Geiger-Müller tube with the two thyroids plated in the centre of a small aluminium planchette.

In all cases the results are expressed as corrected counts per minute (CCPM), together with standard deviation and standard error of the mean where this is applicable. Because of the wide range of values encountered, standard deviation and standard error were calculated on the log-mean.

Calibration of the Veall Tube and Geiger-Müller Tube.

In order to interpret the results obtained in experiments involving correlation of the activity present in liquid and dry samples it was necessary to arrive at a conversion factor by means of which readings obtained using end-window equipment could be converted to compare with those obtained using the Veall tube and vice versa. From a concentrated stock solution of ^{131}I, serial dilutions of known specific activity were made up in 9.0 ml. volumes for counting in the Veall tube (Table XVIIa) and a similar series of standards plated on planchettes and evaporated to dryness for calibration of the end-window counter. The accuracy of the method of preparing the planchettes was confirmed by counting duplicate samples. The calibration curves obtained are shown in Figure 15 and 16. Two counting positions are available in the end-window castle. It was not possible to count the more highly active samples (1 and 2 Table XVIIIb) in "position 1", the position nearer the end-window normally used in counting thyroid material. These were therefore counted in "position 2", at a greater distance from the tube, and samples 3-8 were counted in both positions. A correction factor, derived from the mean value for the ratio $\frac{\text{CCPM in position 1}}{\text{CCPM in position 2}}$ for samples 3 to 8, was then applied in correcting the counts obtained for samples 1 and 2 to position 1. (Table XVIII).

FIG. 15.

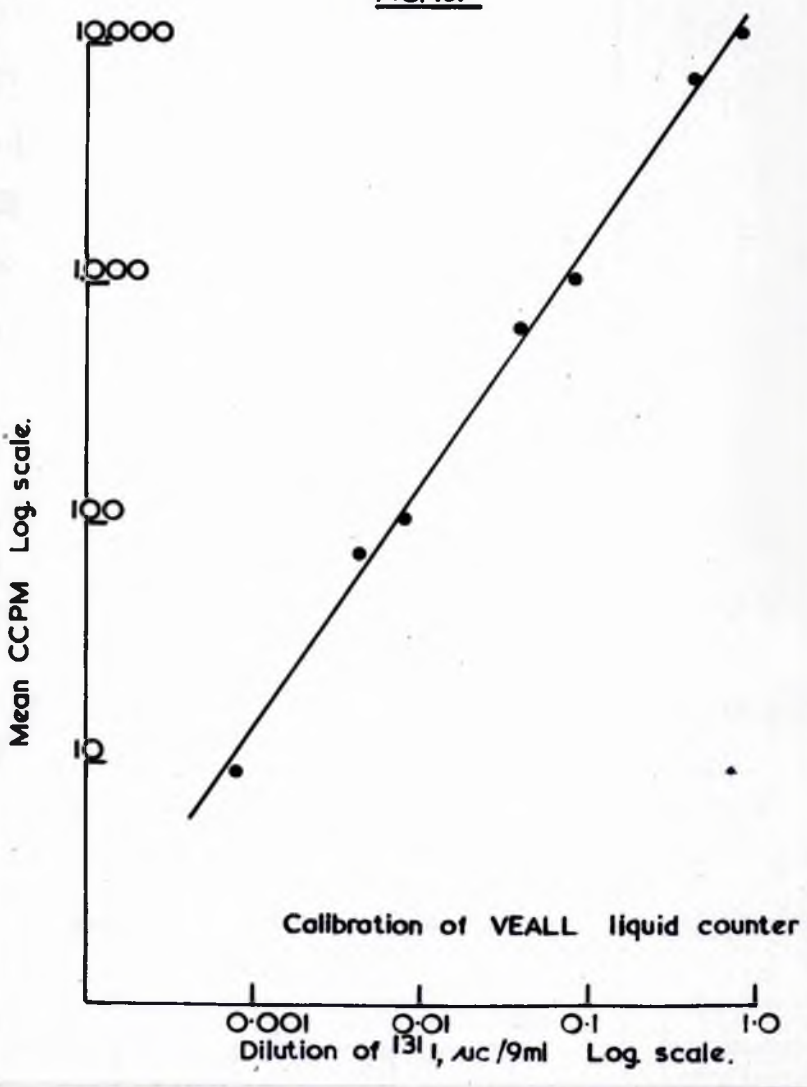

10,000
1,000
100
10
Mean CCPM Log. scale.
Calibration of VEALL liquid counter
0·001
0·01
0·1
1·0
Dilution of 131 I, μc/9ml Log. scale.

TABLE XVIIa

Calibration of Veall tube

Dilution of ^{131}I µc/9.0 ml.	CCPM of sample
0.77	10,855
0.39	5,469
0.077	1,011
0.039	511
0.0077	100
0.0039	57
0.00077	9
0.00039	-

TABLE XVIIb

Calibration of end-window counter

	Number of samples.	Concentration ^{131}I (µc)	Mean CCPM	Counting Position
1.	3	0.39	81,906	2
2.	2	0.077	20,264	2
3.	2	0.039	10,108	2
4.	2	0.039	10,759	1
5.	2	0.0077	2,163	2
6.	2	0.0077	2,281	1
7.	2	0.0039	924	2
8.	2	0.0039	1,064	1
9.	2	0.00077	221	1
10.	2	0.00039	121	1

FIG. 16

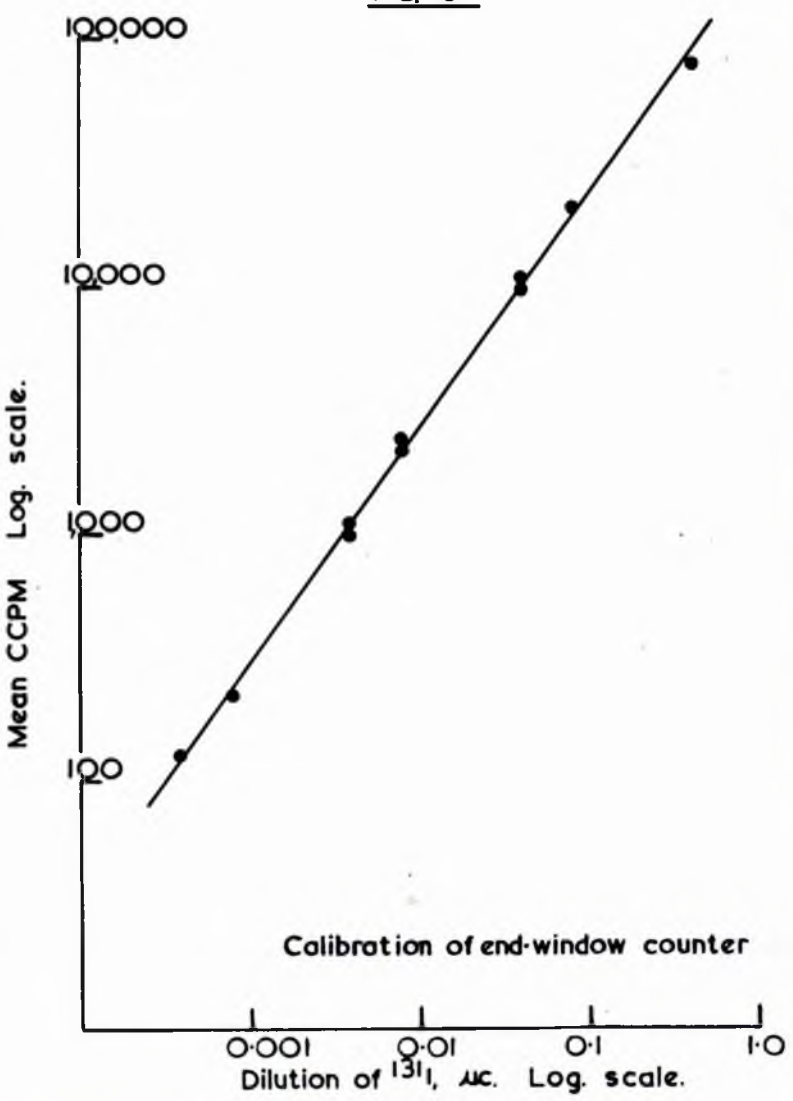

100,000
10,000
1000
100
Mean CCPM Log. scale.
Calibration of end-window counter
0·001
0·01
0·1
1·0
Dilution of 131I, μc. Log. scale.

TABLE XVIII

Correction factor from position 2 to position 1.

CCPM in position 2.	CCPM in position 1.	CCPM in position 1 / CCPM in position 2
4,991	10,712	2.1
5,117	10,807	1.7
1,148	2,344	2.0
1,015	2,218	2.2
483	1,101	2.3
441	1,031	2.1

Mean correction factor = 2.06

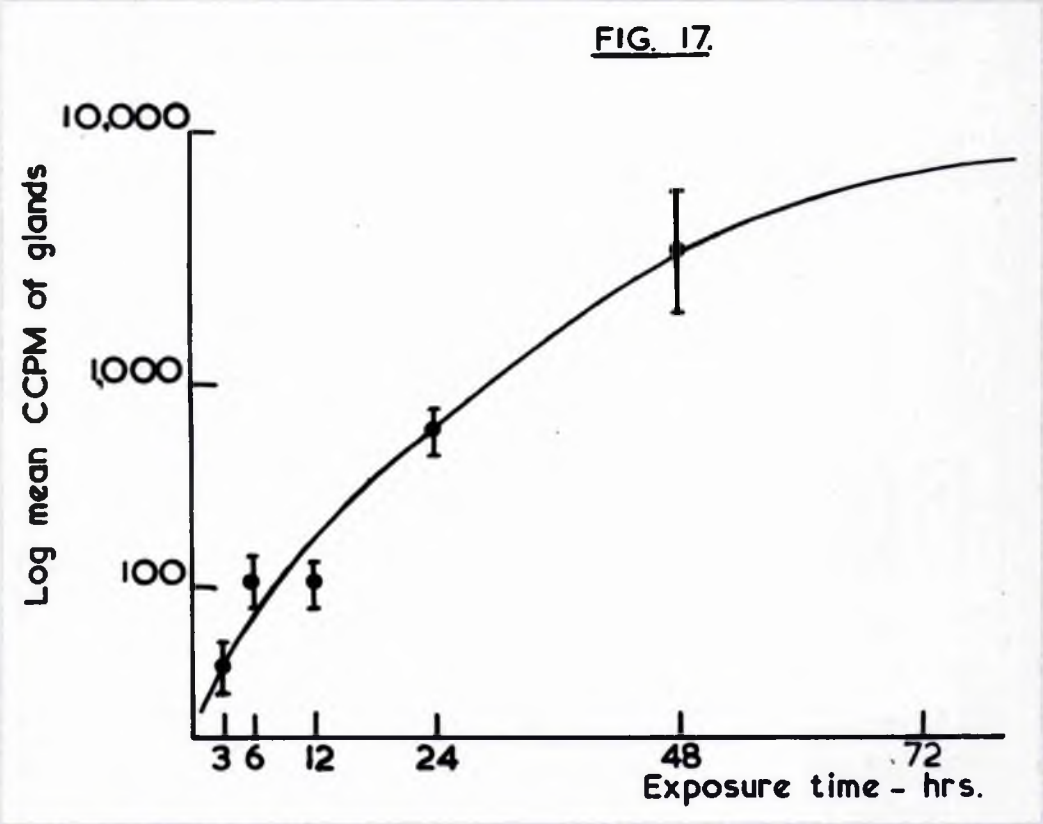


Fig. 17

Increase in ^{131}I accumulation by the thyroid with increase in exposure time.

III. RELATIONSHIP BETWEEN ^{131}I-UPTAKE AND EXPOSURE TIME.

Before investigation of the factors influencing iodine uptake in animals immersed in radioactive iodine, it was necessary to determine the normal pattern of uptake of iodine in untreated animals. Groups of animals were therefore immersed in ^{131}I at a concentration of 20µc/1000 ml., for times varying from 3 to 48 hrs., at 20°C., and the radioactive content of the glands was recorded. (Table XIX).

Results

TABLE XIX

Exposure time (hrs.)		3	6	12	24	48
Mean hind-limb length (mm) range		5.28 3.9-3.0	4.65 6.8-3.1	4.77 6.4-3.1	4.66 6.5-3.5	5.06 5.8-3.2
Number of animals		9	8	9	8	9
Mean CCPM of glands		46.86	113.4	114.8	664.8	3288.0
Log. mean CCPM ±	Standard Deviation	±0.32 1.67	±0.302 2.05	±0.292 2.06	±0.216 2.81	±0.75 3.52
Log. mean CCPM ±	Standard Error	±0.156 1.67	±0.107 2.05	±0.097 2.06	±0.077 2.81	±0.25 3.52

It was necessary to select a suitable exposure time to obtain an iodine uptake giving a convenient counting rate in the end-window castle used in measuring the activity present in the glands. It can be seen (Fig. 17) that the amount of iodine present in the glands increases slowly over a period of 48 hrs. with no clearly defined initial rise. This gradual increase suggests a much slower turn-over of iodine than in the mammals. By extrapolation of

the curve it appears that uptake of ^{131}I would level off after 72 hrs. exposure to iodine. Forty-eight hours was therefore selected as the most useful exposure time for further experiments. The individual variability in iodine uptake was found to be considerable; this problem of variability will be discussed in greater detail in a later section.

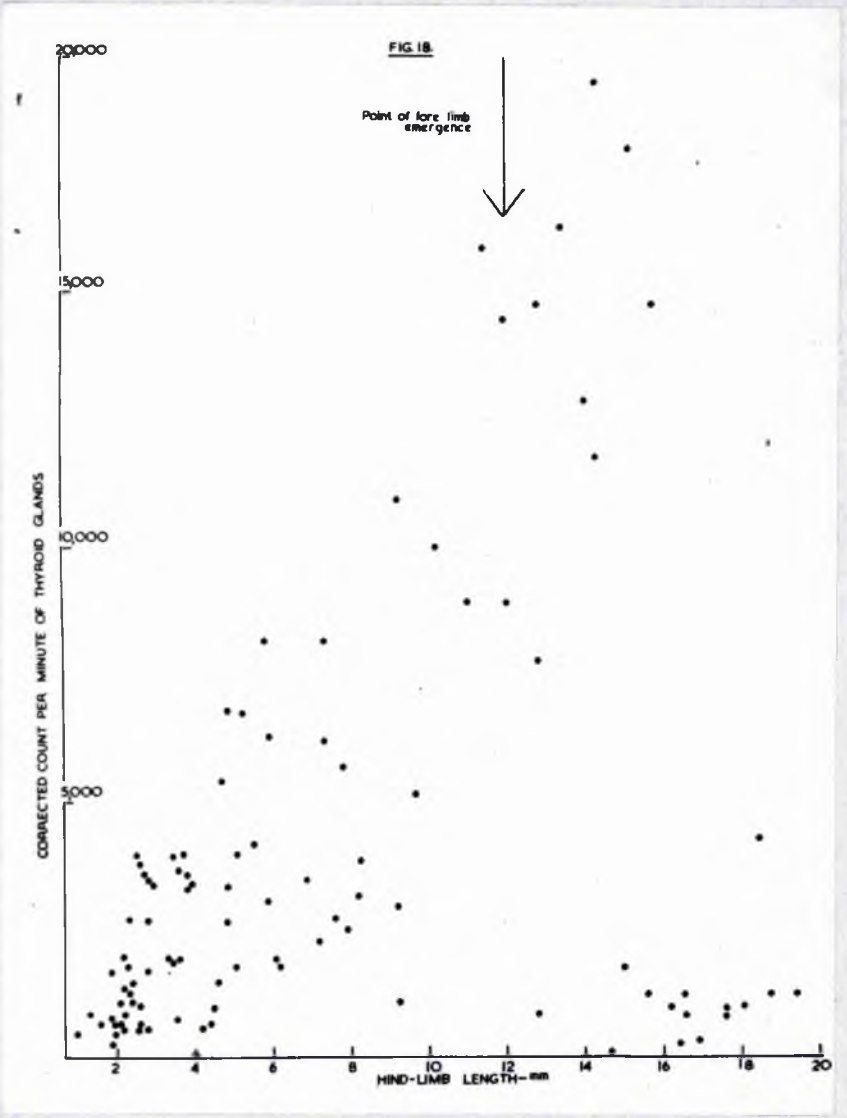


Fig. 18

Thyroidal ^{131}I-uptake in the course of metamorphosis in larvae of Xenopus laevis.

IV. VARIATION IN ^{131}I UPTAKE IN THE COURSE OF METAMORPHOSIS.

The variation in the amount of iodine accumulated by the thyroids in the course of metamorphosis was studied by immersing larvae of all stages of development in ^{131}I, at a concentration of 10μc/1000 ml. of tap water, for 48 hrs. at 20°C. The animals were divided, 10 per jar, so that each jar contained animals of similar size and stage of development.

Staging of larvae.

Workers on amphibian development have recognised that chronological age does not given an accurate measurement of progress in metamorphosis. For iodine uptake studies, increase in hind-limb length was used as the criterion of metamorphic advance in preference to staging according to the 'Normal Table of Xenopus laevis' (1956), since the latter is based upon material collected from natural surroundings. It was found that animals reared under artificial conditions do not conform strictly to the pattern of development on which the "Normal Table" is based.

Results.

The results are presented graphically in Figure 18 and 18a. It can be seen that the amount of activity in the glands increases with increase in hind-limb length, reaching a maximum level at the time of emergence of the fore-limbs. Thereafter, with the onset of shrinkage, change of shape and resorption of the tail, the thyroidal iodine content drops off abruptly. These findings agree closely with thos of Saxèn and his co-workers (Saxèn, Saxèn, Teivonen and Salimaki, 1957a, 1957b) based upon a correlation of iodine accumulation with chronological age in metamorphosing larvae and young toads and published after the present work had been completed.

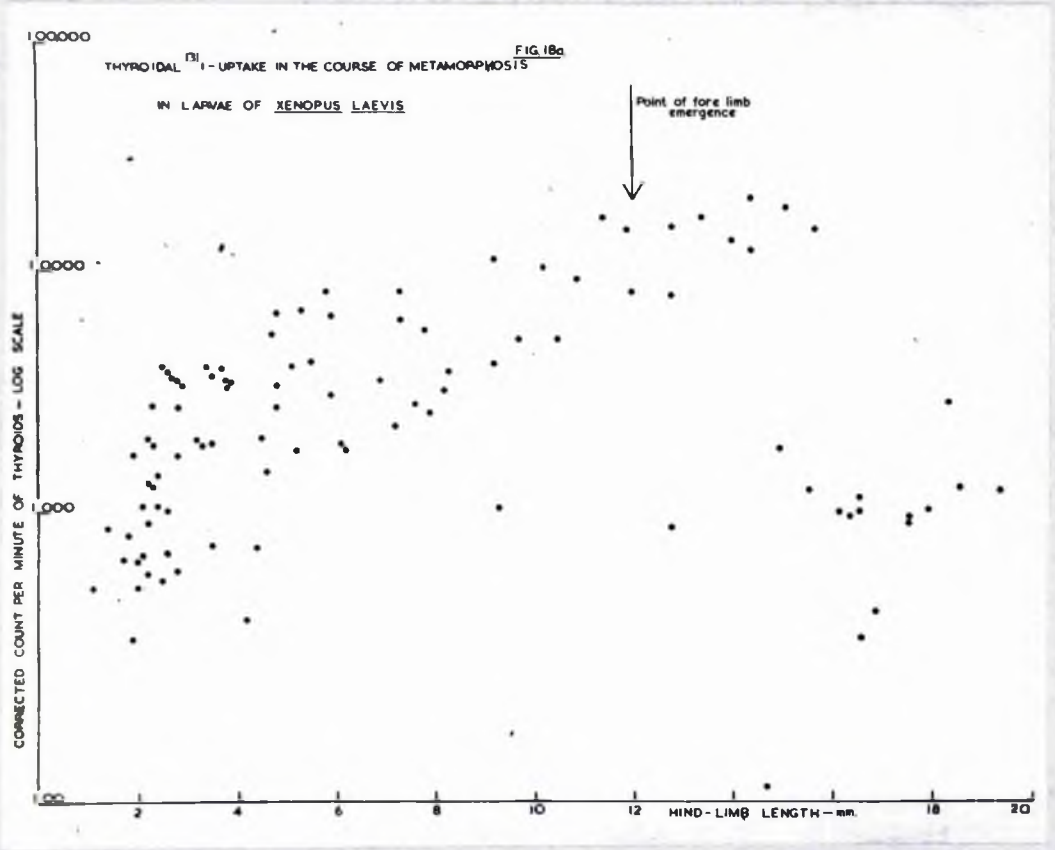


FIG. 18a
THYROIDAL ^{131}I - UPTAKE IN THE COURSE OF METAMORPHOSIS
IN LARVAE OF XENOPUS LAEVIS
Point of fore limb emergence
CORRECTED COUNT PER MINUTE OF THYROIDS - LOG SCALE
100000
10000
1000
100
2
4
6
8
10
12
HIND-LIMB LENGTH - mm.
18
20

The majority of observations on the part played by the thyroid in the course of metamorphosis are based on morphological and histological studies. It was demonstrated by Allen (1919) that, in anurans, the thyroid increases in size up to the peak period of metamorphic activity, after which it gradually decreases. Parallel observations of epithelial hypertrophy and atrophy have been recorded by other workers. With regard to interpretation of these findings, agreement is general that they represent an initial increase in the secretion and accumulation of colloid during the earlier period of hind-limb growth, and subsequent colloid discharge, while change in shape and body shrinkage occur. (Allen, 1938). Further to this, the 'critical period' demonstrated by D'Angelo and his co-workers (D'Angelo, Gordon and Charipper, 1941) in their studies on the effect of starvation on the pituitary-thyroid axis must coincide with the stage at which, as indicated by the earlier workers, iodine accumulation is replaced by colloid discharge.

The changes occurring in iodine accumulation by the thyroid during the course of metamorphosis add further support to these conclusions. The metabolism of iodine by the thyroid may be regarded as an active cycle of uptake and discharge. Up to the time of emergence of the fore-limbs, the iodine relations of the tadpole thyroid are predominantly those of synthesis and storage of the hormone. Emergence of the fore-limbs and the subsequent phases of development are accompanied by discharge of the stored hormone so that glands of recently metamorphosed toads are characterised by a low iodine content.

This process of iodine metabolism, considered in greater detail, can be seen to consist of an initial resting phase followed by an increase in thyroid activity while active metamorphosis is taking place. At early limb-bud stages the thyroidal iodine content is low. Active hind-limb growth

coincides with a marked increase in iodine uptake as the storage of hormone in the thyroid develops. After eruption of the fore-limbs an abrupt change in iodine metabolism occurs. Increased discharge of the stored colloid is demonstrated by a rapid falling off of the activity present in the glands of animals with hind-limb lengths 12 to 18 mm. The low iodine content of the glands of newly metamorphosed toads is thus a direct result of the maximal release of stored iodine in the final stages of metamorphosis. From these observations, it may be concluded that the thyroid hormone, synthesised during the earlier growth period, is mainly utilised in the body during the process of shrinkage and change of shape, and not, as might have been supposed, for fore-limb eruption.

The degree of individual variation in the amount of iodine present in the glands of animals of similar hind-limb length, shown in Figure 18, would require to be greatly reduced before the larvae could be used in assaying TSH by estimation of ^{131}I uptake. Subsequent investigations of the various factors influencing thyroidal iodine metabolism were therefore directed toward establishing a means of controlling this variability in the test animals.

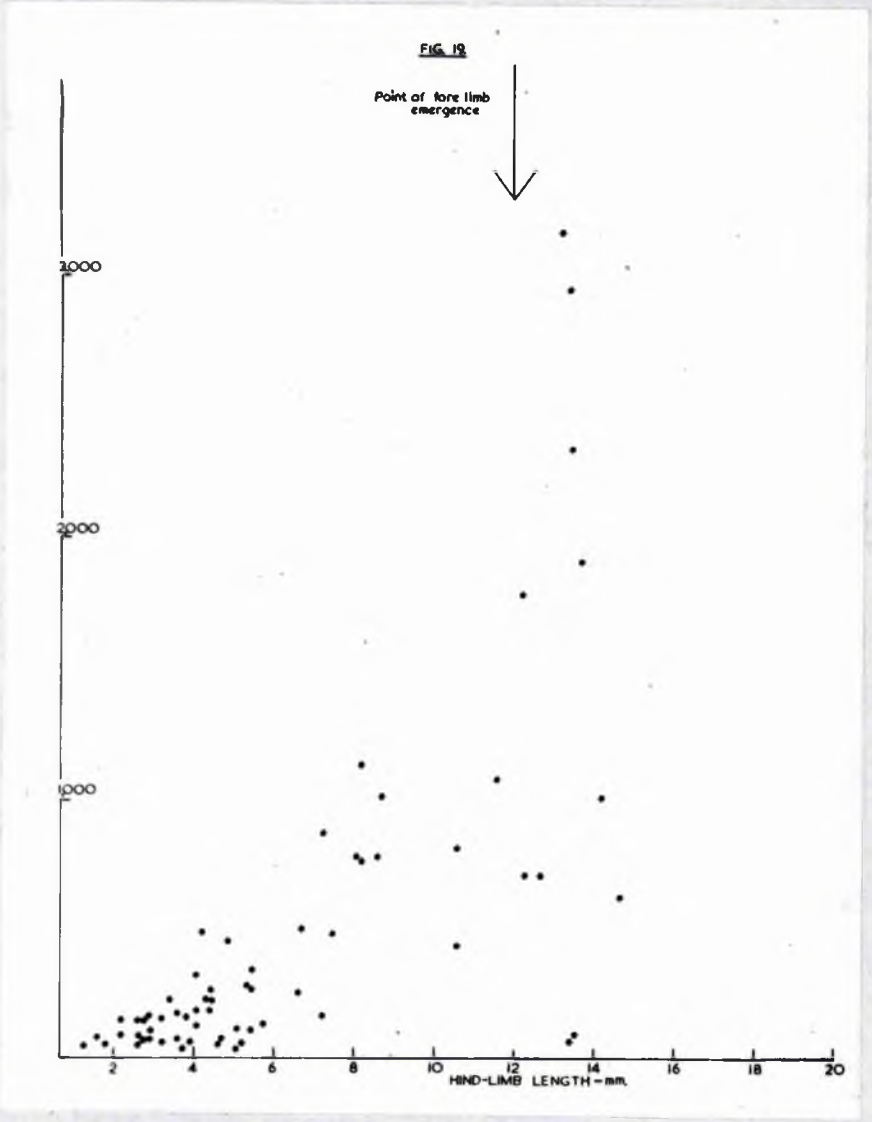


Fig. 19

Thyroidal ^{131}I-uptake in the course of metamorphosis in larvae of Bufo bufo.

V. VARIATION IN ^{131}I UPTAKE IN THE COURSE OF METAMORPHOSIS IN BUFO AND IN RANA TEMPORARIA.

It seemed possible that the individual variability observed in Xenopus larvae might be attributed to the fact that these animals were taken from a population obtained by hormonally induced ovulation and reared under artificial conditions. To investigate this possibility a similar study was carried out on larvae of the common toad, Bufo bufo collected from their natural surroundings and also on a few tadpoles of Rana temporaria.

Larvae of the common toad were collected at an early limb-bud stage and kept in large aquaria in pond water at room temperature. They were fed on weed from the pond from which they were taken. The Rana tadpoles were treated in a similar manner.

Groups of ten larvae were exposed to ^{131}I, at a concentration of 10µc/1000 ml., for 48 hrs. at 15°C. Initially some larvae were exposed to iodine at 21°C as in the earlier experiments on Xenopus, but at this temperature they became inactive and showed signs of stress which were not evident at 15°C. Thyroidectomy was carried out as in the Xenopus larvae. The thyroids did not present variation in size and shape to the extent to which it was observed in the Xenopus larvae, and were uniformly slightly pigmented.

Results.

The results, graphed in Figure 19, 19a and 20, show that in both Bufo and Rana larvae in the earlier stages of metamorphosis, up to the time of eruption of the fore-limbs, the pattern of iodine uptake is similar to that demonstrated in the Xenopus larvae. In the Bufo series there is an

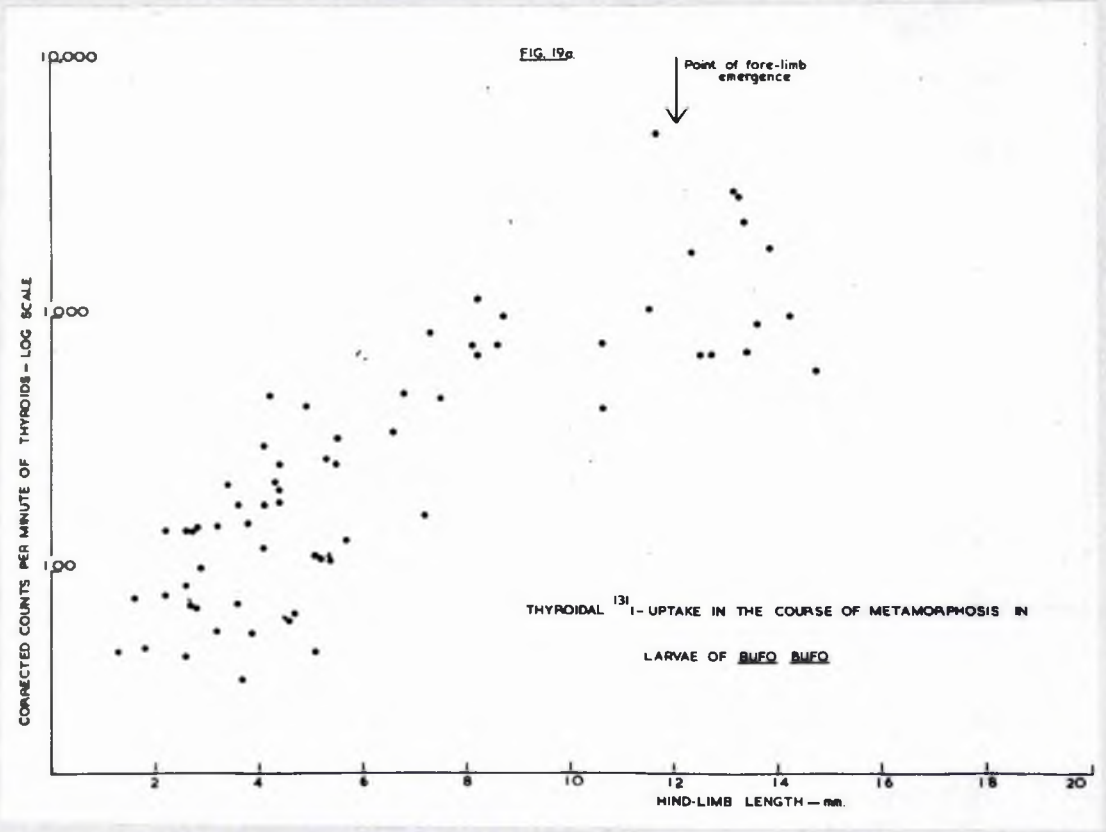


FIG. 19a.
Point of fore-limb emergence
10,000
1,000
100
CORRECTED COUNTS PER MINUTE OF THYROIDS – LOG SCALE
THYROIDAL ^{131}I – UPTAKE IN THE COURSE OF METAMORPHOSIS IN LARVAE OF BUFO BUFO
2
4
6
8
10
12
14
16
18
20
HIND-LIMB LENGTH — mm.

indication of a subsequent falling off in the thyroid iodine level, but no larvae survived through the later stages of metamorphosis.

In all three series of larvae in which ^{131}I uptake during metamorphosis was studied the animals were exposed to ^{131}I at a fixed concentration and for a period of 48 hrs. Variation in both the concentration of iodine to which the animals are exposed and the time for which they are immersed in the solution of ^{131}I should be investigated. In using radioactive tracers to study thyroid metabolism it has been demonstrated that the results are influenced by the time limits of the treatment with iodine as well as by the amount of iodine used. Money, Lucas & Rawson (1955) studied ^{131}I turnover in larvae of Rana pipiens, exposing the larvae to ^{131}I for periods up to 10 days in duration and obtained results different from those recorded above.

A considerable degree of scatter was observed in the Bufo larvae as in the experiment using Xenopus larvae. A comparison of the extent of the variability encountered in the artificially reared Xenopus larvae with that in the Bufo larvae obtained from natural surroundings was made by calculating the correlation between increase in hind-limb length and increase in iodine uptake by the thyroid glands in the two groups of larvae. The correlation coefficient (r) was calculated for the two sets of results from the lowest hind-limb length to hind-limb length 12 mm.

Values for 'r' in the two experiments were found to be:-

Xenopus larvae 'r' = 0.563 Degree of freedom = 82

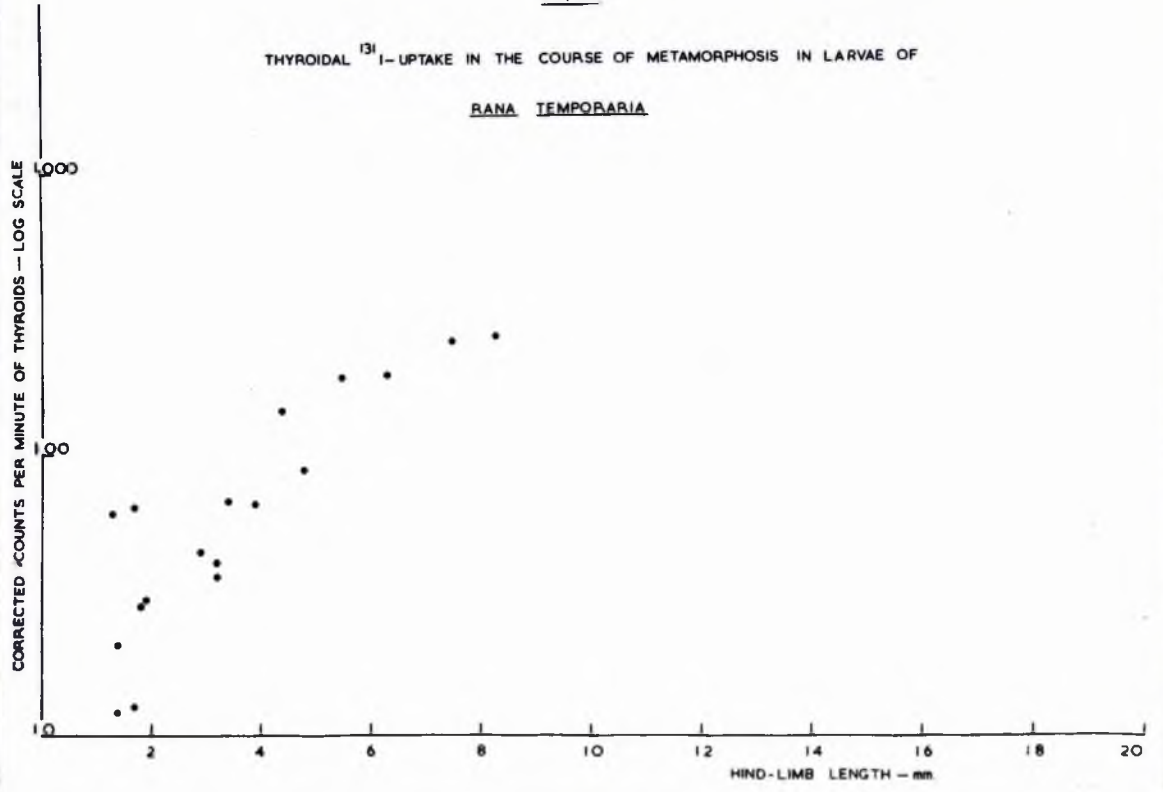
Bufo larvae 'r' = 0.868 Degree of freedom = 55

Correlation is shown to be slightly greater in the Bufo series, but the values obtained for 'r' are such that, with the number of degrees of freedom available in the two experiments, there is no significant difference

FIG. 20.

THYROIDAL ^{131}I – UPTAKE IN THE COURSE OF METAMORPHOSIS IN LARVAE OF

RANA TEMPORARIA



in the degree of correlation between hind-limb length and iodine uptake by the thyroids in the two groups. This is interpreted as indicating that the variability in thyroidal iodine uptake demonstrated in the Xenopus larvae is not due to the artificial conditions under which they are reared since it occurs to an almost similar extent in the Bufo larvae.

VI. DISTRIBUTION OF IODINE BETWEEN THYROID AND OTHER BODY TISSUES

Three experiments were carried out to investigate the distribution between thyroid and other body tissues of iodine administered

a. by injection and b. by immersion

1. Sixteen larvae of long hind-limb length were injected with 0.02 ml. of ^{131}I at four dose-levels, four animals per dose-level, and each group placed in 500 ml. of water. After 6 hrs., the animals were killed, weighed and thyroidectomised. The four groups of glands were pooled on planchettes and their activity determined. The bodies were homogenised individually in 5 ml. of a 50/50 (v/v) mixture of concentrated nitric and sulphuric acids. The homogenate was allowed to stand for 24 hrs. then the volume made up to 9 ml. with water for counting in the Veall tube.

Results.

The relative concentrations of iodine in the body and thyroid were determined by reference to the calibration curves (Figure 15 and 16). The amount of iodine present in the thyroids and in the body homogenate and discharged into the water was calculated as a percentage of the dose administered. (Table XI).

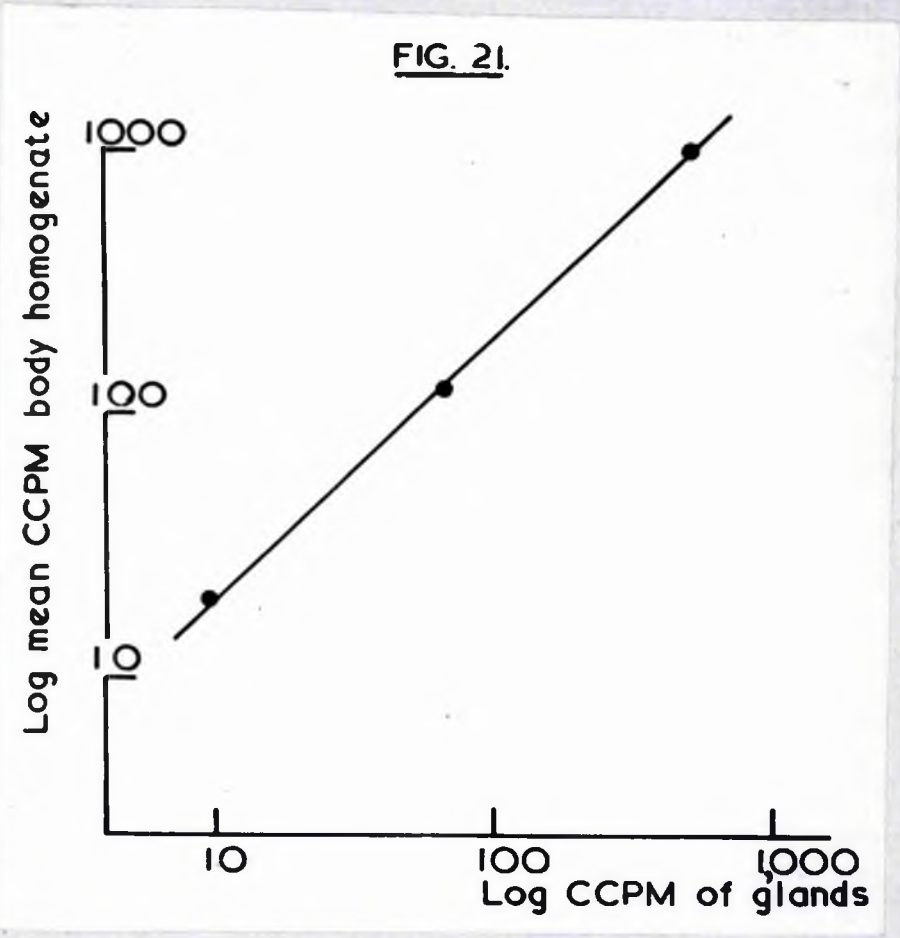


Fig. 21

Correlation between thyroidal ^{121}I accumulation and iodine present in extrathyroidal tissues 6 hrs. after injection of ^{131}I.

TABLE XX

Number of animals	4	4	4	4
Mean hind-limb length (mm.) range	12.4 (11.0-15.2)	11.0 (9.3-13.9)	11.4 (10.1-12.5)	13.1 (11.7-14.3)
Mean body weight (mg.) range	770 (700-830)	723 (600-880)	778 (670-920)	775 (660-850)
Dose ^{131}I, μc/0.02ml.	0.39	0.039	0.0039	0.00039
CCPM pooled glands	2074	266	37.6	6.2
Equivalent Concentration ^{131}I per single gland	0.0019μc	0.00022μc	-	-
% Dose	0.77	0.58	-	-
Mean CCPM of body homogenate	993.2	120.2	19.5	-
Log. CCPM ± standard deviation	±0.44 2.997	±0.126 2.08	±0.182 1.29	-
Mean equivalent Concentration ^{131}I	0.042μc	0.0054μc	0.0014μc	-
% Dose	10.76	21.53	35.89	-
CCPM/9 ml. sample of water	192	17	-	-
Total activity/500 ml.	0.75μc	0.06μc	-	-
% dose.	46.1	38.4	-	-

The counting rates of the pooled glands of the groups of animals which received 0.0039μc and 0.00039μc respectively were of too low an order to obtain values for the iodine content of the individual glands. A direct linear correlation is found to exist between the t corrected counts per minute per gland and the mean corrected counts per minute for the body homogenate (Figure 21). This suggests that the effect

observed is simple adsorption of iodine in both the thyroid and other body tissues. It therefore appears that iodine metabolism by the thyroid can not be demonstrated 6 hrs. after administration of ^{131}I at the low order of concentration employed. These findings could be explained on the basis of a slower turnover rate of iodine in the thyroid glands of the tadpole than is found in the mammalian gland. To determine whether a difference in the ability of the thyroid and other body tissues to concentrate iodine could be demonstrated, a further series of animals was treated with a higher dose of ^{131}I for an increased period of time:-

2. An injection of 1.0 μc ^{131}I in 0.01 ml. was administered intraperitoneally to each of 10 larvae which were then placed individually in 200 ml. of water for 24 hrs. prior to thyroidectomy. The amounts of iodine retained in the glands and discharged into the water were calculated as a percentage of the dose as in the previous experiment. (Table XXI) An increase in percentage uptake of iodine by the glands in comparison with the amount discharged into the water is evident at 24 hrs. after injection, whereas, at 6 hrs. it was not apparent. However, to obtain an accurate estimation of the relative ability of the thyroid and other body tissues to concentrate iodine, it is necessary to make some measurement of gland size. Accordingly, an experiment was performed along lines similar to thos employed in this and the previous experiment and determination of gland weight was attempted:-

TABLE XXI

Hind-limb length (mm.)	Body Weight (mg.)	% Dose in thyroid glands	% Dose discharged into water
13.2	701	5.2	14.9
5.8	670	2.6	6.5
9.4	861	7.9	6.5
10.1	878	7.2	4.3
8.2	748	5.6	5.6
5.8	523	3.0	22.6
12.7	591	6.6	11.8
15.4	551	11.8	24.8
10.9	520	10.8	2.6
9.9	817	8.2	4.1
		mean = 6.89 %	mean = 9.87 %

3. To determine the ability of thyroid tissue to concentrate iodine in comparison with that of other body tissues in the tadpole, it was necessary to arrive at a value for the amount of iodine per unit weight in each case. Because of the small size of the glands, only an approximate figure for the thyroid weight could be obtained using a conventional balance. The procedure used was as follows:-

Six larvae were immersed in 20μc. ^{131}I/1000 ml. of water for 48 hrs. The animals were then killed, weighed and the thyroid glands excised. The

glands were placed on a pre-weighed planchette and weighed, on a microbalance, to the nearest 10 µg. as rapidly as possible to minimise water-loss. The activity in the thyroids was then determined; the body tissues were homogenised as previously described and the activity in the homogenate also determined. The ability of the thyroid tissue to concentrate iodine relative to the other body tissues was then calculated. (Table XXII).

TABLE XXII

Tadpole number	1	2	3	4	5	6
Hind-limb length (mm.)	14.9	4.4	3.5	3.0	3.9	3.9
Body weight (mg.)	580	390	440	360	410	380
Thyroid weight (µg.)	70	30	30	20	30	40
CCPM of body	3703	10043	12587	19321	19895	11267
Total activity in body (µc)	0.25	0.69	0.83	1.26	1.38	0.78
Equivalent activity (µc/mg body weight)	3.42×10^{-7}	1.77×10^{-6}	1.88×10^{-5}	3.49×10^{-6}	3.37×10^{-6}	2.05×10^{-6}
CCPM of thyroids	2944	251	822	954	1092	725
Total activity in thyroids (µc)	0.012	0.00079	0.0032	0.0033	0.0036	0.0024
Equivalent activity (µc/mg thyroid weight)	1.71×10^{-4}	2.63×10^{-5}	1.07×10^{-4}	1.65×10^{-4}	1.16×10^{-4}	5.99×10^{-5}
Ability of thyroid tissue to concentrate iodine relative to that of other tissues	x 500	x 14.3	x 57.0	x 47.25	x 34.4	x 29.0
Mean value for animals	No. 2 - 6 = x 36					

In experiment 1. of this series, it was found that thyroid tissue could not be shown to concentrate iodine at a greater rate than other tissues when ^{131}I was administered at a low concentration and the animals were killed 6 hrs. after injection of the iodine. The amount of iodine retained in the thyroid and other tissues was attributed to simple adsorption. Experiment 2., in which a higher dosage of iodine and a longer period of treatment were used, demonstrated that, after 24 hrs., the proportion of the dose retained in the glands is higher in relation to the amount discharged into the water than is the case after 6 hrs. The findings in these two experiments together suggest that iodine metabolism in the tadpole occurs at a slower rate than in mammals.

The third experiment was designed to determine, on a weight basis, the relative abilities of the thyroid and other tissues to concentrate iodine. Owing to the limited sensitivity of the balance on which the glands were weighed, the figures obtained must be regarded as approximate. It has, however, been demonstrated that in tadpoles of short hind-limb length, 3.0 - 4.4 mm., the ability of the thyroid tissue to concentrate iodine is thirty-six times greater than that of other body tissues. Animal number 1. was further advanced in metamorphosis than the remaining five, with the fore-limbs clearly visible in their pouches and about to erupt. In this animal, the ability of the thyroids to concentrate iodine was increased to 500 times that of the other tissues, more than 10 times greater than in the smaller animals. This order of increase can be correlated with the increase in activity found with advance in metamorphosis in Section IV (page 80). The corrected count per minute of the glands of animals with hind-limb length 12 - 14 mm. was found to be slightly more than 10 times greater than that in animals with hind-limb lengths in the region of 3 - 5 mm.

In this connection it is of interest to note the relative

concentrations of iodine in thyroid and other tissues quoted by Salter (1940) for various mammals. Some examples are given below:-

Iodine concentration γ %	Thyroid	Liver	Skeletal muscle
Man	29,400 - 29,256	118 - 102	30
Dog	19,500 - 8,500	170 - 39	61 - 39
Rabbit	33,000 - 1,170	18 - 2	-

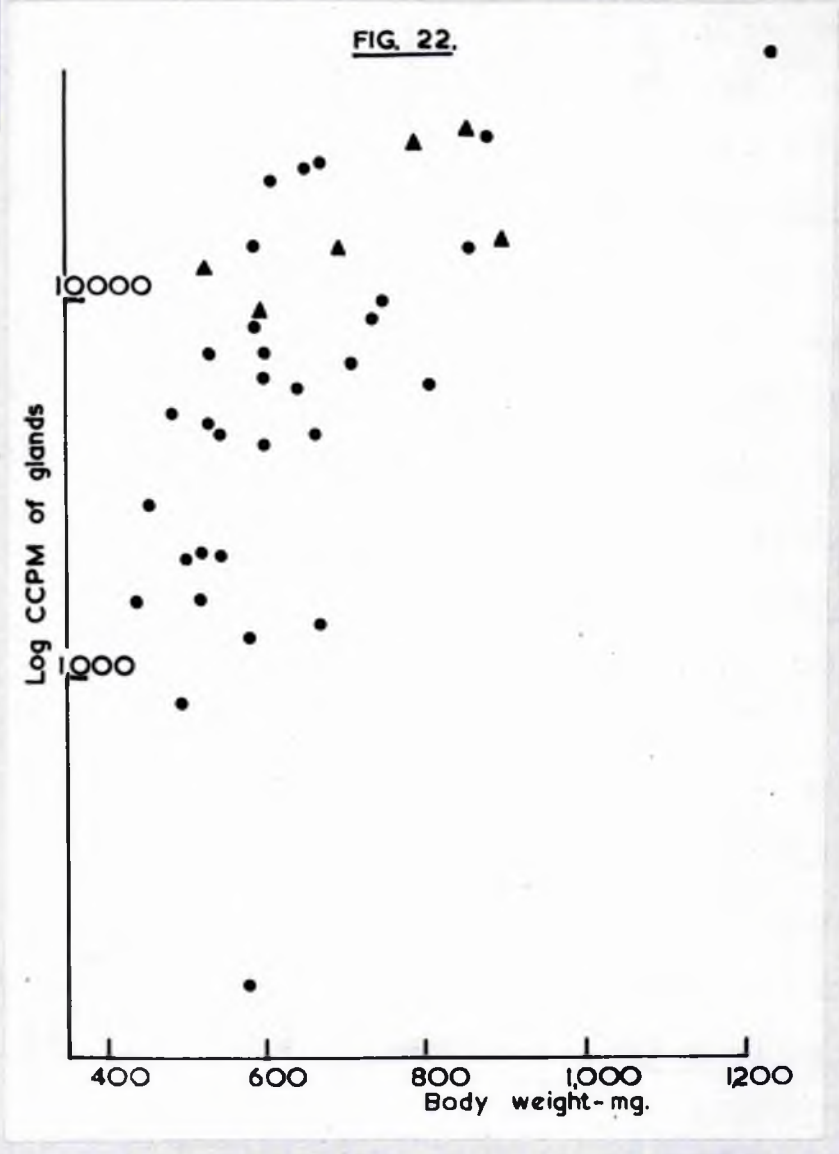


Fig. 22

Variation in thyroidal ^{131}I accumulation with increase in body weight.

▲ denotes tadpoles in which the fore-limbs have erupted.

VII. VARIATION IN ^{131}I UPTAKE WITH INCREASE IN BODY WEIGHT AND THYROID SIZE.

1. Increase in body weight.

Increase in weight has been used, together with chronological age, as a measure of advance in metamorphosis in thyroid studies by Saxén and his co-workers. (Saxén et al. 1957).

The correlation between iodine uptake by the thyroids and increase in weight was studied by exposing a group of larvae to ^{131}I, as in Section IV and weighing each individual before thyroidectomy. From the results obtained (Figure 22) a general trend is discernable in the direction of increased thyroidal iodine uptake with increase in weight up to the time of eruption of the fore-limbs. However, increase in body weight is not a particularly suitable criterion by which to measure metamorphic advance in view of the known facts regarding shrinkage and change of shape in the latter stages of development.

2. Increase in thyroid size.

It was found, in the preceeding section, that determination of thyroid size by weighing individual glands on a conventional balance is not practical because of the small size of the glands. It was therefore necessary to consider an alternative method of assessing thyroid size. The volume of a true elipsoid can be calculated, but the glands cannot be regarded as perfectly symmetrical in shape. When placed in a drop of water on a coverslip the isolated gland naturally orientates itself so that it lies with its largest surface-area parallel to the horisontal plane. An indication of gland size was obtained by an arbitrary measurement of the longest (d_1) and the shortest (d_2) diameters of a gland suspended in this way in 0.75% saline, using a Watson micrometer eye-piece. A "thyroid factor" (T) was then derived from the formula $T = A(d_1 \times d_2) + B(d_1 \times d_2)$ where A and B

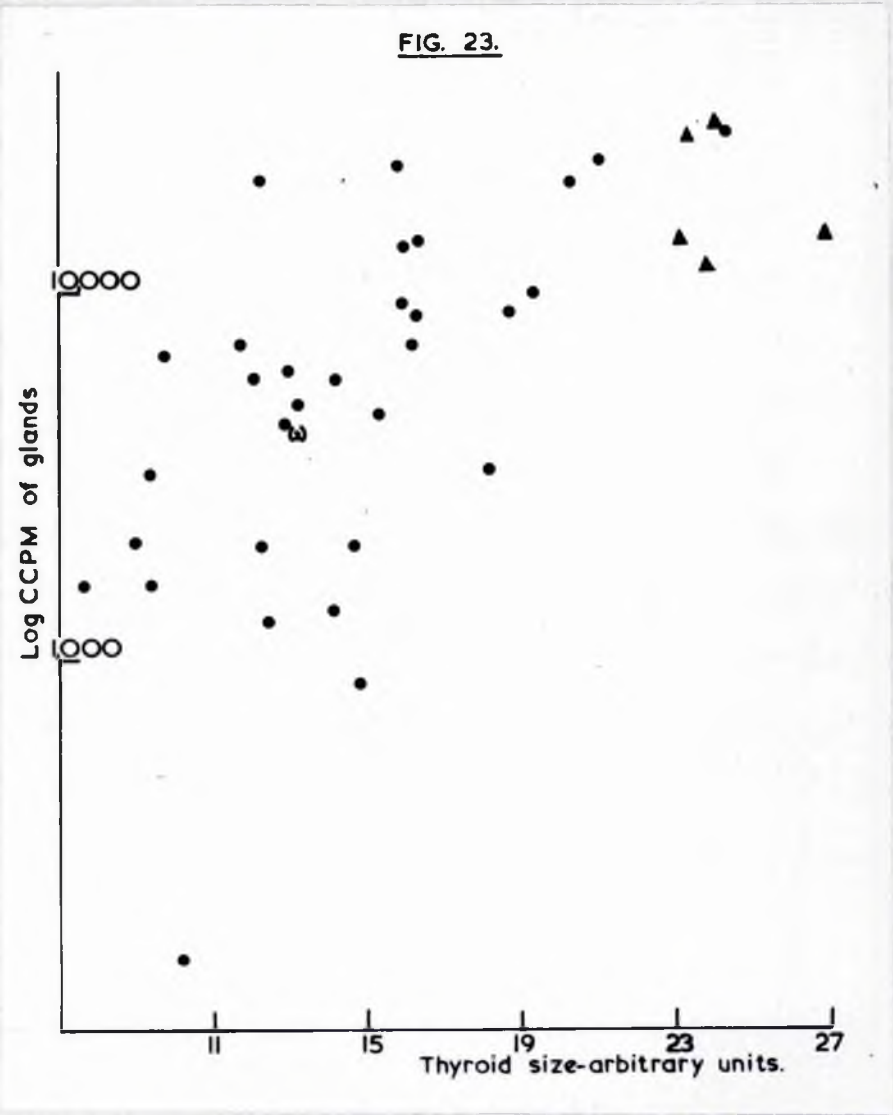


Fig. 23

Variation in thyroidal ^{131}I accumulation with increase in thyroid size.

▲ denotes tadpoles in which the fore-limbs have erupted.

represent the two thyroid lobes. Thus an arbitrary measure of the amount of thyroid tissue present in each individual was obtained. The "thyroid factor" was correlated with the amount of iodine present in the glands in a series of larvae exposed to ^{131}I at a concentration of 10 μc/1000 ml. for 48 hrs. (Figure 23). The expected trend of increase in accumulated ^{131}I with increase in gland size is demonstrated. The scatter observed must be partially attributed to the approximate method used to determine gland size.

Increase in accumulation of ^{131}I by the thyroids appears to be directly correlated both with increase in body weight up to the time of eruption of the fore-limbs and increase in gland size. In view of the change in shape and weight-loss which occur in the final stages of metamorphosis, weight increase is a poor criterion by which to measure metamorphic advance beyond the peak of metamorphic activity. By the method employed, an approximate measurement of the largest cross-section of the gland and hence an indication of gland size was obtained. It would be necessary to employ a more elaborate technique for estimation of gland size before an accurate determination could be made of the correlation between the amount of thyroid tissue present and the rate of accumulation of iodine.

VIII. THE EFFECT OF DIET ON ^{131}I UPTAKE

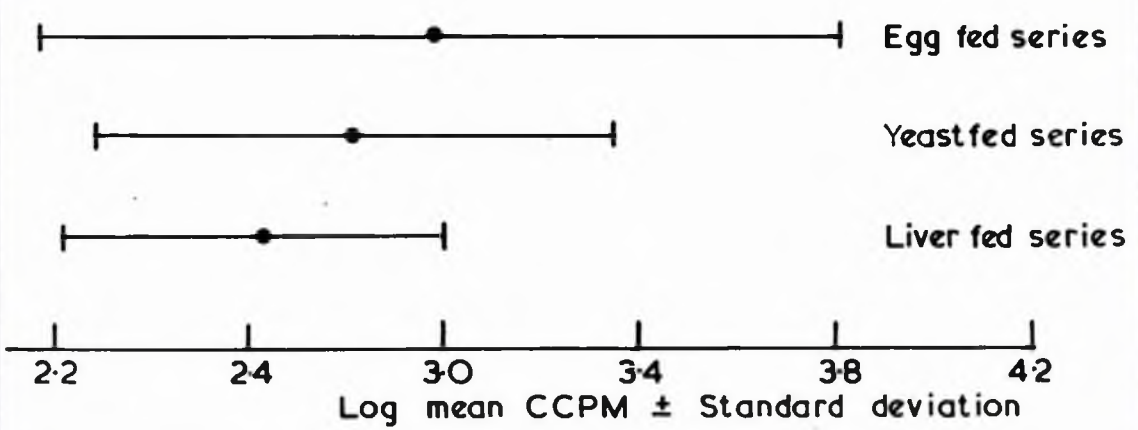
In the course of the preceeding investigations of ^{131}I accumulation during metamorphosis and in relation to gland size and body weight, it has become evident that a very large variability in iodine uptake by the thyroids can occur in an apparently uniform group of larvae. It has been shown that this individual variability occurs to an equal extent in animals reared under artificial conditions and animals collected from their natural surroundings. The diet on which the larvae are reared is a major factor in controlling the rate of growth and metamorphosis. It is therefore to be expected that the food-stuffs used might influence the degree of variability in iodine accumulation, chiefly depending upon the fact that onset and progress of metamorphosis are determined by the amount of iodine ingested in the food.

Various food-stuffs have been used by different workers. Those more commonly employed have been a suspension of boiled spinach or nettle (Money et al 1955, Jurand, 1955), dried yeast (Asboe-Hansen et al 1952) or liver powder (Dodd & Landgrebe, 1953). The amount of iodine available in these different food-stuffs varies considerably. In the section on animal husbandry it was stated that the routine employed in rearing the larvae was followed with the aim of reducing individual variability to a minimum and producing large, metamorphically retarded animals suitable for assay work. With this object in view, three food-stuffs, egg-powder, liver-powder and dried yeast were selected for investigation.

Groups of 10 larvae were selected from large batches of animals which had been reared on egg, liver and yeast respectively for a period of eight weeks from the time of hatching. Each group of 10 animals was exposed to ^{131}I at a concentration of 10μc/1000 ml., for 48 hrs. at 21°C.

FIG. 24.

Effect of diet on thyroidal ^{131}I accumulation



Results.

TABLE XXIII

	Mean hind-limb length (mm.)	Number of animals	Mean CCPM	Log mean CCPM ± standard deviation
Egg fed series (50 mg./3 days)	3.29 (2.8 - 4.1)	10	968.3	2.00 ±0.819
Yeast fed series (50 mg./3 days)	2.89 (2.3 - 4.1)	10	645.7	2.81 ±0.533
Liver fed series (50 mg./3 days)	3.51 (2.7 - 4.6)	10	436.5	2.64 ±0.324

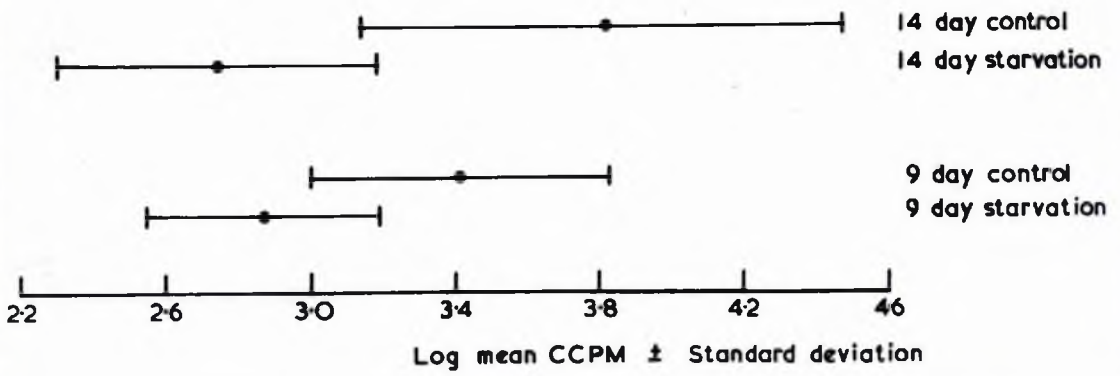
The greatest variability was shown to occur, together with the highest uptake of iodine, in the egg-fed series (Table XXIII. Figure 24). A slightly smaller degree of variability occurred in the liver-fed group than in the yeast-fed group. Since onset of metamorphosis depends largely on the amount of iodine ingested and liver has a relatively high iodine content, animals fed exclusively on liver tend to metamorphose more rapidly and at a smaller size than animals reared on a diet with a lower iodine content. Yeast-fed animals were found to be slow growing, reaching a larger body size with relatively short hind-limbs.

On the basis of the findings recorded above, egg powder was discarded as unsuitable for routine rearing purposes since it produced an uneven growth rate within a batch of animals and a high degree of variability in iodine uptake in a uniform group of animals selected from the egg-fed population. Variability was found to occur to a lesser extent in the liver-fed than in the yeast-fed

series, while the latter animals achieved a greater size with short hind-limb length, more suitable for assay work. Accordingly, a 50/50 mixture of these two food-stuffs was adopted for routine rearing purposes. On this diet, the growth-rate was somewhat slower than on liver alone and more uniform groups of animals were obtained of a size suitable to withstand handling. The variability in iodine uptake appeared to be reduced as compared with that which occurred in animals fed on yeast alone.

FIG. 25.

Effect of starvation on thyroidal ^{131}I accumulation.



II. THE EFFECT OF STARVATION ON ^{131}I UPTAKE.

The thyroid epithelium in the "stasis" tadpole used in the histometric method, presents a uniformly squamous appearance. The iodine uptake of glands in which an atrophic condition had been induced in this way was studied using 40 larvae. Four groups of 10 larvae of uniform size were selected. Two of these groups were subjected to starvation periods of nine and fourteen days respectively. The other two control groups were fed 50 mg./ 3 days during this time. At the end of the starvation period the "stasis" group and control group were exposed to ^{131}I at a concentration of 10μc/1000 ml., for 48 hrs. prior to thyroidectomy.

Results.

TABLE XXIV

	Mean hind-limb length (mm.)	Number of animals	Mean CCPM	Log. mean CCPM ± standard deviation
9-day "stasis" group	4.8 (2.1 - 6.9)	9	741	2.87 ±0.324
9-day control group	8.1 (4.2 - 10.6)	9	2,570	3.41 ±0.406
14-day "stasis" group	6.6 (3.0 - 14.4)	10	550	2.74 ±0.439
14-day control group	11.7 (6.9 - 15.0)	8	6,457	3.81 ±0.657

A barely significant reduction in variability is shown by the 9-day "stasis" animals (Table XXIV, Figure 25). But in both the 9-day and 14-day "stasis" groups the amount of iodine accumulated by the glands is greatly

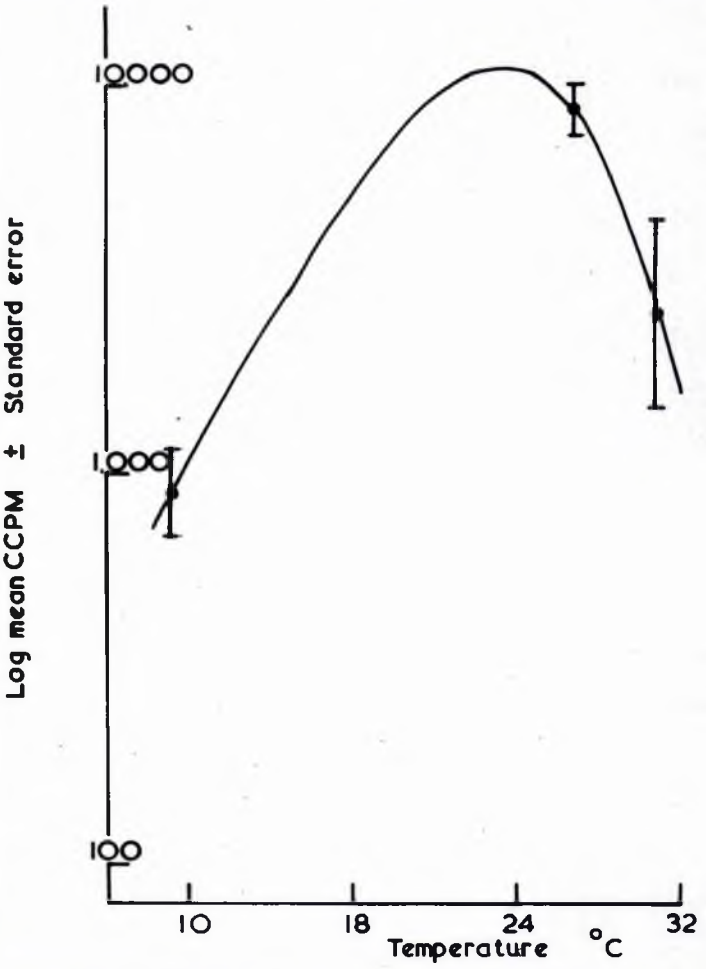
reduced. In the 9-day group the mean uptake in the starved animals is 29% of that of the control group and in the 14-day group it is further reduced to 8.5% demonstrating that the glands have been reduced to an almost completely inactive state.

Although variability is only slightly less in the "stasis" group than in the fed group, the lowered iodine content of the glands in the starved animals suggests that, as in the histometric method, in assaying TSH by iodine uptake "stasis" animals should be used. With the glands in an atrophic state and therefore a low level of iodine uptake in the untreated animal, any increase in iodine uptake occurring in response to administered TSH should be more clearly defined than in fed animals where the unstimulated level of uptake is very much higher.

In the animals starved for a period of 14 days, effects of starvation other than on the functional status of the thyroid become evident, in particular marked shrinkage and reduced viability. It is doubtful whether animals starved for this length of time would withstand a series of injections in the course of an assay.

FIG. 26a.

Effect of temperature on thyroidal ^{131}I accumulation



I. THE EFFECT OF TEMPERATURE ON ^{131}I UPTAKE

The important part played by the pituitary and thyroid glands in the course of amphibian metamorphosis is further complicated by the influence of temperature on the degree of functional activity of these glands. It has been well established that metamorphosis can be accelerated or retarded by increasing or lowering the temperature at which the larvae are maintained.

Two series of larvae were used in studying the effects of temperature variation on ^{131}I uptake by the thyroids:-

1. Three groups of larvae were exposed to ^{131}I at a concentration of 10μc/1000 ml., for 48 hrs. at 31°, 29° and 9°C respectively. (Table XXVa, Figure 26a.)
2. Five groups of larvae were exposed to ^{131}I of the same strength as in 1. for 48 hrs. at 31°, 27°, 22°, 15° and 8°C. (Table XXVb, Figure 26b.)

Results.

TABLE XXVa

Temperature (°C)	Mean hind-limb length (mm.)	Number of animals	Mean CCPM	Log. mean CCPM ± standard deviation
31	8.6	10	2559	3.408 ±0.757
27	8.9	10	8472	3.928 ±0.218
9	8.7	9	881	2.945 ±0.319

FIG. 26b.

Effect of temperature on thyroidal ^{131}I accumulation

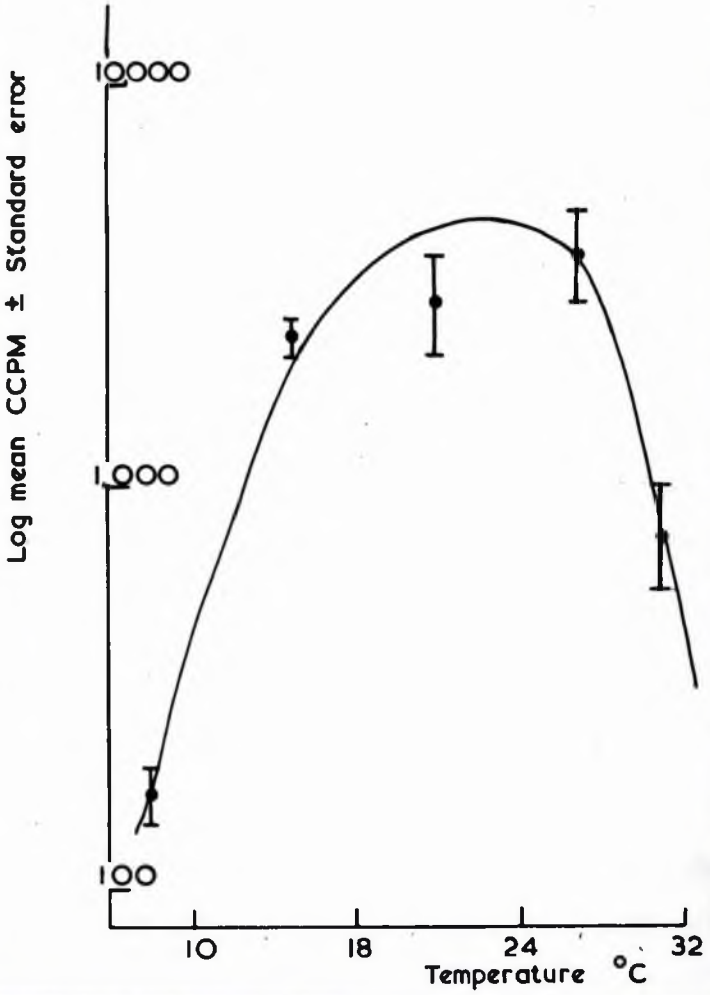


TABLE XXVb.

Temperature (°C)	Mean hind-limb length (mm.)	Number of animals	Mean CCPM	Log. mean CCPM ± standard deviation
31	6.8	8	738	2.868 ±0.377
27	6.4	10	3724	3.571 ±0.344
22	6.1	10	2812	3.449 ±0.368
15	5.5	10	2328	3.367 ±0.128
8	5.9	9	191	2.280 ±0.219

A reduction in the amount of iodine taken up by the glands is shown to occur in all the groups exposed to extremes of temperature. The animals exposed to temperatures 31°, 9° and 8°C became pale and inactive. This pallor may be compared with that seen in the "stasis" larvae in which thyroid atrophy is secondary to a state of pseudohypophysectomy. The melanophores in these animals assume a punctate condition and fail to give the normal response of expansion when the larvae are placed on a dark background. This failure to respond to change in background is interpreted as being indicative of pituitary inactivity and failure of the secretion of intermedin together with other pituitary hormones. It is suggested that the pallor exhibition by the animals exposed to extremes of temperature is similar in origin to that observed in the "stasis" larvae; these animals also remained pale on a dark background. It is possible that pituitary failure is caused by exposure to extremes of temperature. Reduction in the amount of iodine taken up by the thyroids would then occur as a secondary effect.

In the intermediate range of temperatures, uptake of iodine is highest between 22 and 28°C. Variability was found to be less in the group exposed at 15°C than in the other groups.

The majore purpose in conducting this investigation on the effect of temperature on iodine uptake was to determine whether individual variability could be influenced by temperature with a view to making use of the effect in the development of an assay method. On the basis of the finding that variability appeared to be less at 15°C than at 21°C, succeeding experiments involving iodine uptake estimations were carried out at the lower temperature. It has been established that temperature plays a critical part in the control of growth and metamorphosis of amphibian larvae. This fact is further emphasised by the findings recorded above, in that the pituitary and hence, the thyroid appear to be inactivated by extremes of temperature. It is therefore suggested that a more detailed examination of this aspect of iodine metabolism in the tadpole would yield material of interest in connection with the endocrine changes occurring during metamorphosis.

XI. DISCUSSION AND CONCLUSIONS

It was found that iodine was accumulated rather slowly by the thyroids in animals immersed in a dilute solution of ^{131}I. A 48 hour exposure period was selected for routine use in uptake studies.

The pattern of iodine uptake during the course of metamorphosis was shown to consist of a gradual rise in the amount of iodine accumulated in the glands up to the time of eruption of the fore-limbs, followed by a decline to the original level in the newly metamorphosed toad, i.e. the greatest accumulation of ^{131}I in the thyroid coincides with the peak period of metamorphic activity, and falls off in the latter stages of development during which colloid storage is replaced by colloid discharge. The individual variability was found to be considerable. This variability can not be attributed to the artificial conditions under which ovulation is induced and the larvae are reared since an almost equal degree of variability was observed in Bufo larvae collected from their natural surroundings.

Although a trend in the direction of increased ^{131}I uptake in relation to increase in body weight up to the time of eruption of the fore-limbs was also demonstrated, too much weight should not be attached to the correlation of these two aspects of development. Shrinkage and weight loss occur in the final stages of metamorphosis and body weight at a given stage is largely dependent upon the type of food-stuff on which the larvae are reared.

An approximate measure of the amount of thyroid tissue present, obtained by estimating the area of the largest cross-section of the glands, indicated a correlation between the increase in the amount of ^{131}I accumulated and the increase in thyroid size. This method of measuring gland size was attempted as an alternative to weighing the glands which was found to be

unsatisfactory.

The amount of iodine present in the glands six hours after injection of a low dose of ^{131}I was found to be small, bearing direct relationship to the amount present in a homogenate of the body. These findings would seem to indicate that there was no differential accumulation of iodine by the glands 6 hrs. after the start of treatment. 24 hrs. after injection of a higher dose of ^{131}I, the amount accumulated by the glands was shown to be greatly increased. It appears that iodine metabolism occurs at a slower rate in the tadpole than in mammals.

An attempt was made to estimate the rate of concentration of iodine on a quantitative basis by weighing the glands and expressing the iodine content as micro-curies per milligram wet weight of tissue. This method of recording gland size was subject to considerable error because of the extremely small size of the glands and loss of water during the process of weighing. To obtain an accurate result it would be necessary to use some means of determining gland size other than the conventional balance, such as a micromethod for determining volume by displacement. In this experiment it was found that the ability of the thyroid to concentrate iodine, as compared to that of the rest of the body, was ten times greater in an animal at the peak of metamorphic activity than in animals at early hind-limb stages. This finding agrees with the earlier observation that the amount of iodine present in the glands is approximately ten times greater at this stage than at the early limb-bud stages or at the completion of metamorphosis.

A high degree of variability between the iodine uptake of animals at the same stage of development was demonstrated throughout the studies on iodine uptake in the course of metamorphosis and its relationship to gland size and body weight. This variability must necessarily be reduced before an assay method for TSH dependent on estimation of increase in uptake of iodine

can be developed. The influence of diet and temperature on variation in iodine uptake was accordingly investigated.

The effect of diet on iodine metabolism is largely dependent upon the amount of iodine ingested in the food. Of three food-stuffs investigated, egg-powder was found to produce animals in which the growth rate was uneven and the variability high. Animals fed on liver powder showed rapid metamorphosis and reduced variability as a result of the relatively high iodine content, while those fed on yeast had a slower growth-rate and reached a larger body-size at early limb-bud stages. For routine rearing purposes a diet consisting of a 50/50 mixture of liver and yeast was therefore selected as being the most effective in producing large, metamorphically retarded animals in which individual variation in iodine uptake was reduced to the greatest possible extent.

Exposure to iodine at extremes of temperature caused marked reduction in thyroid activity; this was attributed to inactivation of the pituitary. In the intermediate range of temperature studied, it was found that iodine uptake was least variable at 15°C, without there being any marked reduction in the amount of iodine accumulated. The observed effects of temperature variation on iodine metabolism are of interest in connection with the influence of temperature on growth rate and metamorphosis in amphibian larvae in general.

Starvation was shown to produce a reduction in iodine uptake. This is to be expected as a result of thyroid atrophy in the "stasis" larva. A reduction in variability was also observed in animals starved for a period of nine days.

It can be seen that variability in iodine uptake is not greatly influenced by either diet or temperature. Controlled conditions of husbandry, however, combined with careful selection of animals for uniformity of both body size and hind-limb length, are essential in investigation of possible

assay techniques for estimation of TSH.

The most suitable conditions for measurement of TSH by stimulation of increase in ^{131}I uptake are those in which the iodine uptake in the untreated animal is low. These conditions prevail in larvae in the early growth stages of metamorphosis, when the hind-limb length is less than 8 mm. and secretion of endogenous TSH is low. Pituitary function in such animals can be further supressed by starvation and a clearly defined response of increase in iodine uptake should be obtained after administration of TSH.

Assay of TSH dependent on estimation of discharge of ^{131}I requires that, initially, the amount of iodine taken up by the unstimulated glands should be high. The animals best suited for use in this type of estimation are therefore those which are at the peak of metamorphic activity in which administration of TSH should result in an increase in the rate of discharge of the stored colloid.

Whether the assay technique employed be dependent on estimation of increase in uptake or discharge of ^{131}I, variability in iodine metabolism in the test animals is a major obstacle to obtaining a good degree of sensitivity and precision. The assay techniques investigated are described in the following sections, together with possible methods of controlling variability other than by regulation of diet and temperature.

PART IV

ASSAY OF TSH BY ESTIMATION OF ^{131}I-UPTAKE

PART IV

ASSAY OF TSH BY ESTIMATION OF ^{131}I-UPTAKE

I. INTRODUCTION.

With the exception of the work reported by Querido et al (1953) assay methods dependent on estimation of ^{131}I-uptake described in the literature have been insufficiently sensitive to be applied in studies on serum TSH levels. The effect on sensitivity of various forms of pretreatment of the test-animals has been investigated by several groups (Querido et al., 1953, Overbeek et al., 1953, Levey et al., 1956). Attempts have also been made to compensate for poor sensitivity by concentration of the TSH in serum by the Cohn method of fractionation (Lamelijer, 1956) and by zone-electrophoresis (Postel, 1956).

All previous studies on thyroid function in the anuran tadpole, using radioactive tracers, have been dependent on estimation of ^{131}I-uptake. Money et al.(1955) demonstrated that the thyroid in Rana pipiens larvae concentrated between 10 and 20 percent of the available radioactivity. They found that pretreatment with TSH usually resulted in a decrease in the amount of ^{131}I retained in the glands except when the radioactive iodine was administered shortly after the TSH was injected. On the other hand, D'Angelo (1956) obtained a significant increase in accumulation of ^{131}I in the thyroids of Rana clamitans larvae after administration of 6 imu. of TSH.

In their investigations of ^{131}I-uptake, Money et al (1955) used periods of treatment with ^{131}I of varying lengths, up to 10 days. They were unable to demonstrate any difference in the rate of ^{131}I-turnover between metamorphosing and non-metamorphosing tadpoles and concluded from

their findings that use of ^{131}I, under the conditions which they employed, would be of little value as a method for assay for TSH. D'Angelo (1956), however, administered ^{131}I, parenterally a short time before killing the test-animals. He obtained a measurable increase in the uptake of both ^{131}I and ^{32}P after injection of TSH and, on the basis of these findings, suggested that this type of estimation might be applicable as an assay method. The conflicting results obtained by these two groups emphasise the importance of the time-relations of treatment when ^{131}I is employed in studying changes induced in thyroid metabolism.

Design of the assay.

The rationale which forms the basis for the selection of animals of hind-limb length 2 - 6 mm. for study of ^{131}I-uptake in response to TSH stimulation has been discussed above. Although it was also shown in the preceeding investigations that a period of starvation prior to treatment with ^{131}I has little effect on individual variation in uptake, this was employed in assay work in order to reduce uptake to a low level in the untreated animals. Response to injected TSH should be more clearly defined in "stasis" larvae in which the thyroid is in a resting condition.

Money et al (1955) studied ^{131}I-uptake in larvae immersed in a dilute solution of ^{131}I and in larvae in which ^{131}I was administered parenterally. In the investigations to be described, results obtained with administration of ^{131}I by immersion, as in the previous experiments, were compared with those obtained after injection. In the immersion experiments the time-relations of the treatment with radioactive iodine were investigated. Animals were exposed to ^{131}I for a period after the final injection of TSH and throughout the injection period.

TSH was administered by injection in a volume of 0.02 ml. The

routine usually followed was a single injection per day. In two experiments, however, twice daily injections were given in an attempt to obtain a more rapid response and thus improved sensitivity.

It was found in the preceeding investigations that neither diet nor temperature had any marked effect in reducing individual variability in iodine uptake. The possibility of controlling variability by pretreatment with iodine has therefore been investigated. The animals were immersed in dilute solutions of iodine in potassium iodide, potassium iodide alone and thyroxine. The effectiveness of this treatment was investigated prior to and during the period in which TSH was injected.

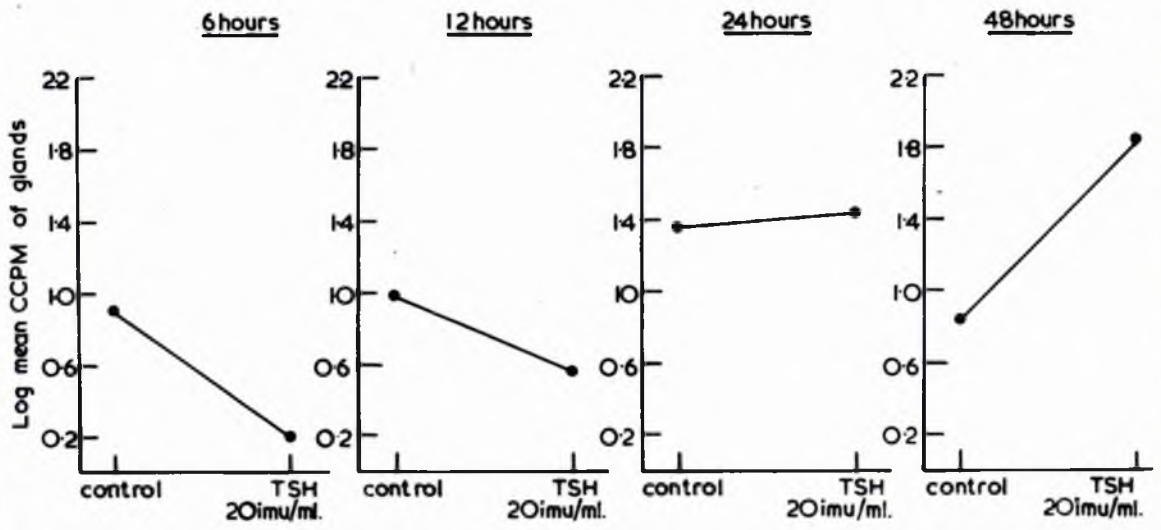
II. UPTAKE OF INJECTED IODINE IN RESPONSE TO TSH STIMULATION

Two experiments were carried out to determine the effect of pre-treatment with TSH on the thyroidal accumulation of injected ^{131}I. The iodine content of the glands of animals pretreated with TSH was compared with that of saline - injected control animals at increasing time intervals up to 72 hrs. after injection of the ^{131}I.

1. Twenty-four animals were divided into groups of 3; two injections of TSH, at a concentration of 20 imu/ml., were given on successive days, followed by an injection of ^{131}I, at a concentration of 0.25 μc/ml., on the third day. Control animals were injected with 0.02 ml. of 0.75% saline. The animals were killed and then thyroids removed at increasing time intervals after injection of ^{131}I from 6 to 48 hrs. The glands of each group of three animals were pooled on a single planchette and their activity determined.

FIG. 27.

Response to TSH at increasing time intervals in animals injected with ^{131}I



Routine:-

Number of animals = 24.

Starvation period = 7 days

Injection schedule day 1. 0.02 ml. TSH - 20 imu/ml.

day 2. 0.02 ml. TSH - 20 imu/ml.

day 3. 0.02 ml. ^{131}I - 0.25 µc/ml.

Results

TABLE XXVII

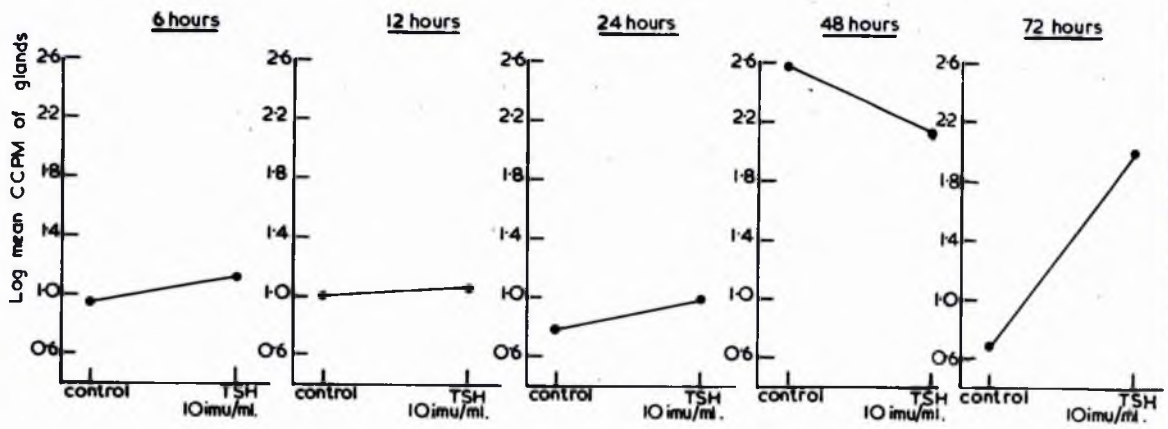
	TSH-injected	Control
Time after injection of ^{131}I = 6 hrs.		
Number of animals.	3	3
Mean hind-limb length (mm.)	5.8	8.7
Mean CCPM of glands	1.6	8.1
Time after injection of ^{131}I = 12 hrs.		
Number of animals.	3	3
Mean hind-limb length (mm.)	6.0	8.5
Mean CCPM of glands	3.7	9.6
Time after injection of ^{131}I = 24 hrs.		
Number of animals	3	3
Mean hind-limb length (mm.)	7.1	8.0
Mean CCPM of glands	26.9	23.1
Time after injection of ^{131}I = 48 hrs.		
Number of animals	3	3
Mean hind-limb length (mm.)	9.6	8.6
Mean CCPM of glands	70.1	7.0

From the figures recorded in Table XXVII it appears that no response to TSH becomes apparent earlier than 48 hrs. after administration of ^{131}I. A second experiment was carried out along similar lines to those above, using a longer injection period prior to administration of TSH.

Groups of 4 animals were used and the glands from each group pooled on a single planchette. Four daily injections of TSH, at a concentration of 10 imu/ml., were given, followed by ^{131}I, at a concentration of 0.25 μc/ml., on the fifth day. The animals were killed at increasing time-intervals up to 72 hrs. after injection of the ^{131}I.

FIG. 28.

Response to TSH at increasing time intervals in animals injected with ^{131}I



Routine:-

Number of animals = 40

Starvation period = none

Injection schedule days 1-4 0.02 ml. TSH - 10 imu/ml.

day 5 0.02 ml. ^{131}I - 0.25 µc/ml.

Results.

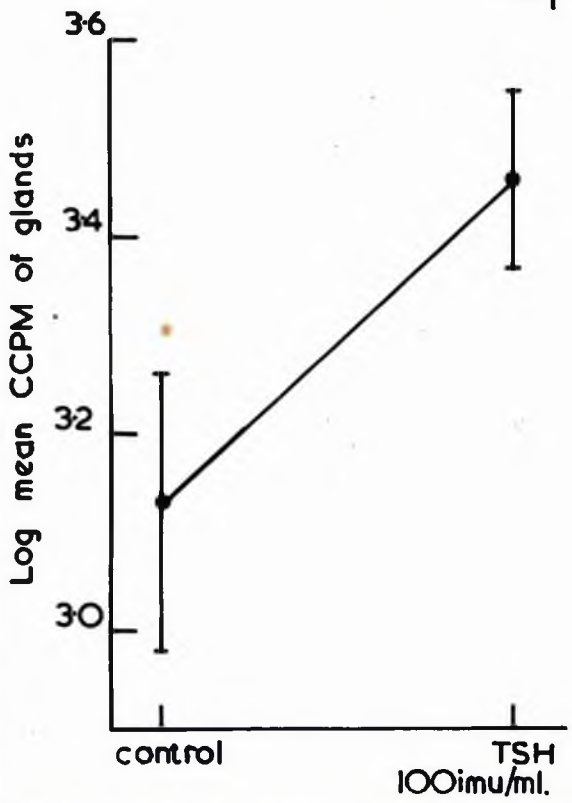
TABLE XXVIII

	TSH-injected	Control
Time after injection of ^{131}I = 6 hrs.		
Number of animals	4	4
Mean hind-limb length (mm.)	7.8	7.0
Mean CCPM of glands	13.0	9.0
Time after injection of ^{131}I - 12 hrs.		
Number of animals	4	4
Mean hind-limb length (mm.)	6.5	5.7
Mean CCPM of glands	11.6	10.0
Time after injection of ^{131}I - 24 hrs.		
Number of animals	4	4
Mean hind-limb length (mm.)	4.5	3.5
Mean CCPM of glands	9.7	6.0
Time after injection of ^{131}I - 48 hrs.		
Number of animals	4	4
Mean hind-limb length	8.0	9.5
Mean CCPM of glands	134	371
Time after injection of ^{131}I = 72 hrs.		
Number of animals	4	4
Mean hind-limb length	7.7	6.8
Mean CCPM of glands	1000	49

The results recorded in Table XXVIII appear to indicate a discharge effect at 48 hrs. after injection of ^{131}I. It is clear from the conflicting results obtained in these two experiments that the time-relations of the response to TSH must be explored with extreme care, together with the effects of using varying concentrations of ^{131}I. Money et al. (1955) obtained an apparent discharge of ^{131}I in response to TSH in some of their investigations and an uptake response in others. D'Angelo (1956) has since reported that he was able to demonstrate increase in iodine uptake in animals of short-hind-limb lengths after administration of TSH; the animals used above had hind-limb lengths in the 7 - 8 mm. range and hence may have been slightly less sensitive. The dose of ^{131}I which D'Angelo administered was 0.5 - 1.0 µc. per animal as compared with 0.005 µc per animal in those experiments. The response-time which he selected was 3 - 4 hrs. after administration of iodine at which time no response to TSH was evident in the above investigations. From these findings it seems probable that the response to TSH can be demonstrated equally successfully either as an uptake or a discharge effect, depending on the conditions of treatment employed.

FIG. 29.

Effect of a single massive dose of TSH on ^{131}I uptake.



XXI. RESPONSE TO TSH STIMULATION IN ANIMALS IMMERSED IN RADIOACTIVE IODINE

1. A pilot experiment was carried out to determine whether any response could be obtained to TSH in animals immersed in radioactive iodine. A single massive dose of TSH was given and the animals exposed to ^{131}I, at a concentration of 20 μc/1000 ml., for 24 hrs. before thyroidectomy.

Results.

TABLE XXIX

	TSH injected (100 imu/ml.)	Control
Number of animals	10	10
Mean hind-limb length (mm.)	4.8 (4.1 - 5.6)	5.3 (4.4 - 6.0)
Mean CCPM of glands	2,875	1,343
Log. mean CCPM ± standard deviation	3.46 ±0.297	3.13 ±0.48
Log. mean CCPM ± standard error	3.46 ±0.094	3.13 ±0.152

A clearly significant response in increase of ^{131}I uptake was demonstrated in the experimental group (Table XXIX, Figure 29). Further experiments were therefore carried out on starved animals given multiple injections and immersed in iodine, to determine the lowest dose to which a measurable response could be obtained.

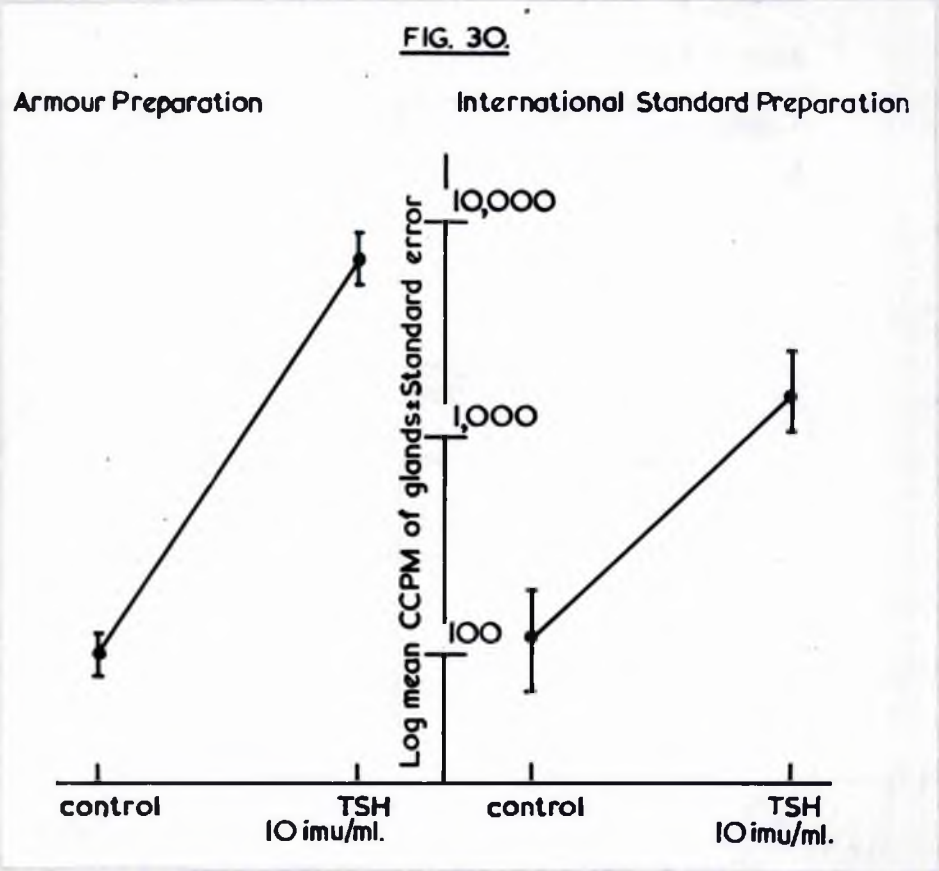


Fig. 30

^{131}I accumulation in response to administration of 10 imu/ml. of TSH.

2. Two thyrotrophin preparations were used in an experiment designed to determine the order of increase in ^{131}I-uptake obtained when animals were immersed in ^{131}I after injection of TSH over a period of five days. The International Standard preparation for TSH and Armour's thyrotrophin preparation were made up at a concentration of 10 imu/ml. and the potencies of the two preparations compared.

Routine:-

Number of animals = 40

Starvation period = 12 days

Injection schedule = 0.02 ml/day/5 days.

^{131}I immersion = 20:c/1000 ml. for 48 hrs. following last injection.

Results.

TABLE XXX

	Control	Armour TSH (10 imu/ml)	Control	Standard TSH (10 imu/ml.)
Number of animals	9	9	7	9
Mean hind-limb length (mm.)	1.9 (0.9-2.7)	4.2 (2.9-5.2)	2.3 (1.4-5.0)	2.3 (1.3-3.0)
Mean CCPM of glands	108	6,751	120	1,678
Log. mean CCPM ± standard deviation	$2.03^{\pm 0.291}$	$3.83^{\pm 0.336}$	$2.08^{\pm 0.608}$	$3.22^{\pm 0.54}$
Log. mean CCPM ± standard error	$2.03^{\pm 0.097}$	$3.83^{\pm 0.112}$	$2.08^{\pm 0.230}$	$3.22^{\pm 0.18}$

It was found that a significant response was obtained to both the Standard and the Armour preparations (Table XXX, Figure 30).

The mean corrected count per minute of the glands of animals injected

with the Armour preparation was approximately four times that of the group given the Standard preparation. The potency of the Armour preparation thus appears to be four times greater than that quoted by the manufacturers.

3. To obtain an indication of the lower limit of sensitivity of this type of estimation, a third experiment was performed in which TSH was injected at concentrations of 1.0 imu/ml. and 0.1 imu/ml.

Routine:-

Number of animals = 30

Starvation period = 14 days

Injection schedule = 0.02 ml/day/6 days

^{131}I immersion = 20 uc/1000 ml; for 48 hrs. following last injection.

Results

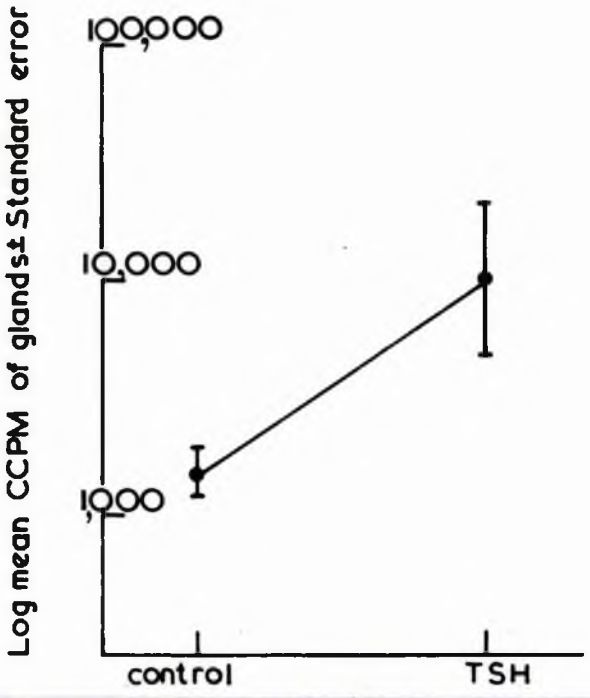
T A B L E XXXI

	TSH injected (1.0 imu/ml.)	TSH injected (0.1 imu/ml.)	Control
Number of animals	10	8	4
Mean hind-limb length (mm.)	3.8	3.7	3.3
Mean CCPM of glands	399	221	512
Log. mean CCPM ± standard deviation	$2.60^{\pm 0.38}$	$2.34^{\pm 0.19}$	$2.71^{\pm 0.512}$
Log. mean CCPM ± standard error	$2.60^{\pm 0.12}$	$2.34^{\pm 0.067}$	$2.71^{\pm 0.256}$

Unfortunately, only 4 of the control animals survived the full injection period. The results of this experiment therefore, were not significant.

FIG. 31.

Response to TSH in animals immersed in ^{131}I throughout injection perjod.



4. In this experiment the effect of giving twice daily injections of TSH was investigated. The animals were exposed to radioactive iodine throughout the injection period.

Routine:-

Number of animals = 10

Starvation period = 5 days

Injection schedule = 0.02 ml. twice daily/3 days.

^{131}I immersion = 20.c/1000: throughout injection period.

Results.

TABLE XXXII

	TSH injected (20 imu/ml.)	Control
Number of animals	5	5
Mean hind-limb length (mm.)	7.1 (5.8 - 7.9)	5.4 (3.9 - 9.0)
Mean CCPM of glands	10,348	1,471
Log mean CCPM ± standard deviation	4.013 ±0.707	3.17 ±0.247
Log mean CCPM ± standard error	4.013 ±0.317	3.17 ±0.111

The results recorded in Table XXXII show that the counting rate of the glands of both the treated group and the control group is higher than in previous experiments as a result of immersion of the animals in the ^{131}I solution throughout the injection period. The amount of iodine accumulated by the treated group, given twice daily injections of TSH at a concentration of 20 imu/ml. for 3 days, was approximately seven times greater than that taken up by the control group. In Experiment 2., the treated group received daily injections of TSH at a concentration of 10 imu/ml. for five days and the amount of iodine accumulated

was more than ten times greater than that taken up by the control group. This would appear to indicate that the order of response obtained is not increased when the number of injections is increased. The absolute amount of TSH administered in Experiment 2. was 1.0 imu and in Experiment 4., 1.2 iu D'Angelo (1956) also found that the minimal effective dose of TSH required to induce an increase in thyroidal ^{131}I accumulation was of the same order of magnitude with both single and multiple injections.

These three experiments (2-4) demonstrate that it is possible to obtain an increased ^{131}I uptake in response to doses of TSH at a concentration of 10 imu/ml. and more. The findings also emphasise the fact, which was evident from studies of iodine uptake without TSH stimulation, that the variability in individual response is a serious obstacle to demonstrating stimulation of iodine uptake by TSH at low orders of concentration. The following experiments were therefore designed to investigate whether variability could be controlled.

FIG. 32.

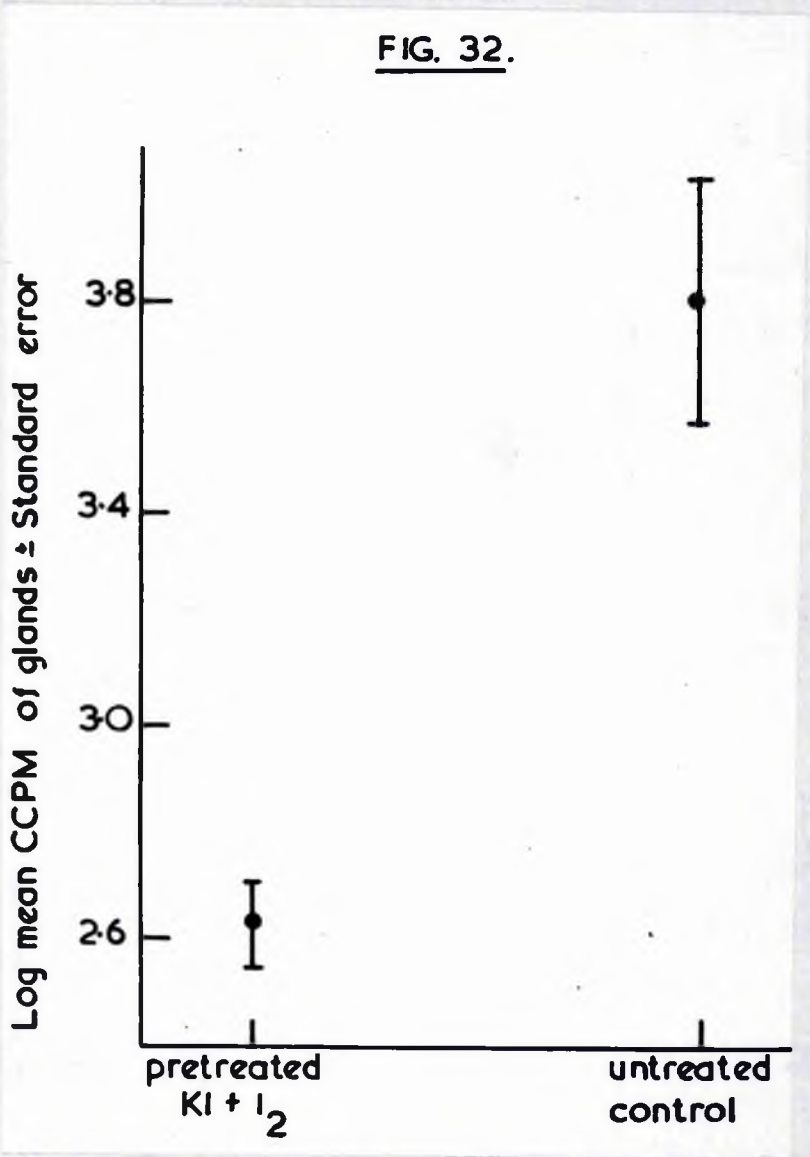


Fig. 32

The effect of pretreatment with iodine in potassium iodide on variation in ^{131}I-uptake.

IV. RESPONSE TO TSH STIMULATION IN ANIMALS TREATED WITH IODINE AND EXPOSED IN RADIOACTIVE IODINE.

1. The effect of exposure of unstimulated larvae to iodine prior to immersion in ^{131}I.

The effect of exposure to iodine prior to immersion in ^{131}I was first investigated without injection of TSH. A group of 10 larvae were immersed in a dilute solution of iodine in potassium iodide for 72 hrs. then transferred to ^{131}I, at a concentration of 20µc/1000 ml. for 48 hrs. The iodine uptake was compared with that of a control group immersed in ^{131}I without pre-treatment. A stock solution of 1% ^{127}I in 2% KI was made up and 0.2 ml. of this added to 1,000 ml. of tap water in which the experimental group were placed.

Results.

TABLE XXXIII

	Pretreatment with iodine.	No pretreatment
Number of animals	10	10
Mean hind-limb length (mm.)	6.9 (3.9 - 15.2)	6.2 (3.5 - 10.4)
Mean CCPM of glands	422	6,349
Log. mean CCPM ± standard deviation	2.63 ±0.263	3.80 ±0.736
Log. mean CCPM ± standard error	2.63 ±0.083	3.80 ±0.233

Treatment with a dilute solution of iodine in potassium iodide was found to produce a considerable reduction in individual variation (Table XXXIII, Figure 32). A marked reduction in iodine uptake was also observed in the pretreated group, the glands having been partially saturated with iodine prior to exposure to ^{131}I.

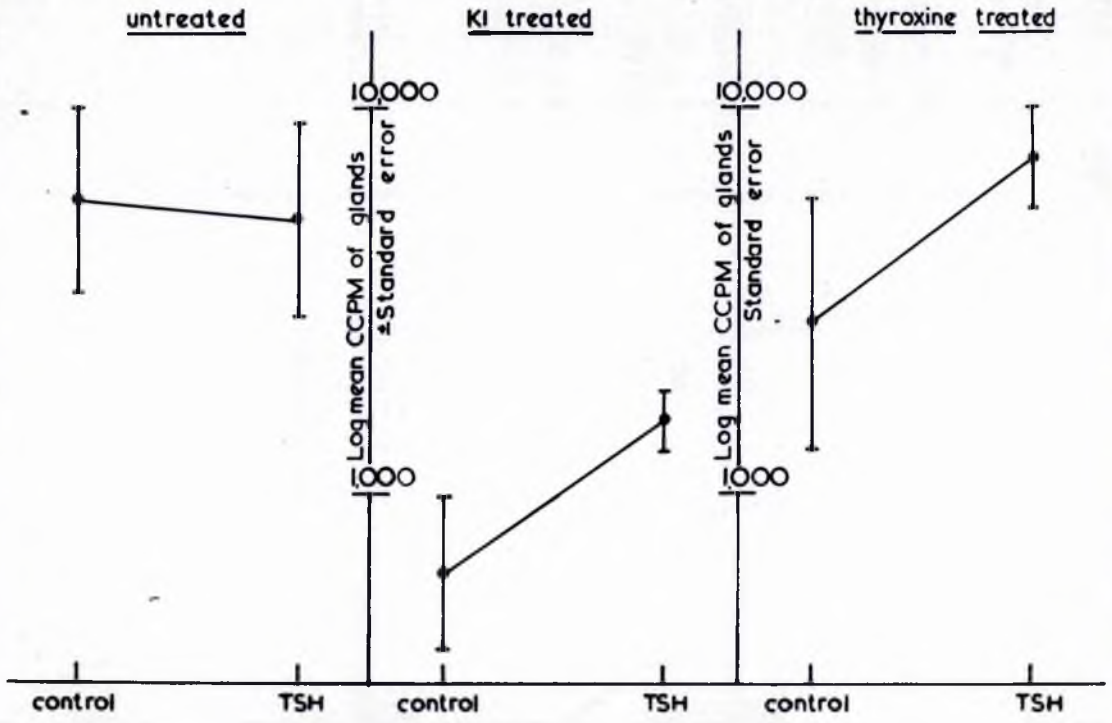
2. Response to TSH stimulation in animals pretreated with:-

a) Potassium iodide and b) Thyroxine

Six groups of eight animals each were starved for a period of 12 days. During the last 48 hrs. of the starvation period, two groups were immersed in a solution of potassium iodide at a concentration of 60γ/1000 ml. and two groups in thyroxine at a concentration of 1 part in 10^9; the last two groups remained in tap water. The experimental groups were then given TSH at a concentration of 10 imu/ml., daily for 5 days prior to immersion in ^{131}I for 48 hrs; the control groups were injected with 0.7% saline.

FIG. 33.

Response to TSH in animals pretreated with potassium iodide and thyroxine



Routine:-

Number of animals = 48 (8 per test group)

Starvation period = 12 days

Pretreatment = 48 hrs. prior to injection period.
a) In potassium iodide; 60γ/1000 ml.
b) In thyroxine: 1 part in 10^9.

Injection schedule = 0.02 ml./day/5 days.

^{131}I immersion = 10μc/1000 ml; for 48 hrs. after the last injection.

Results.

TABLE XXXIV

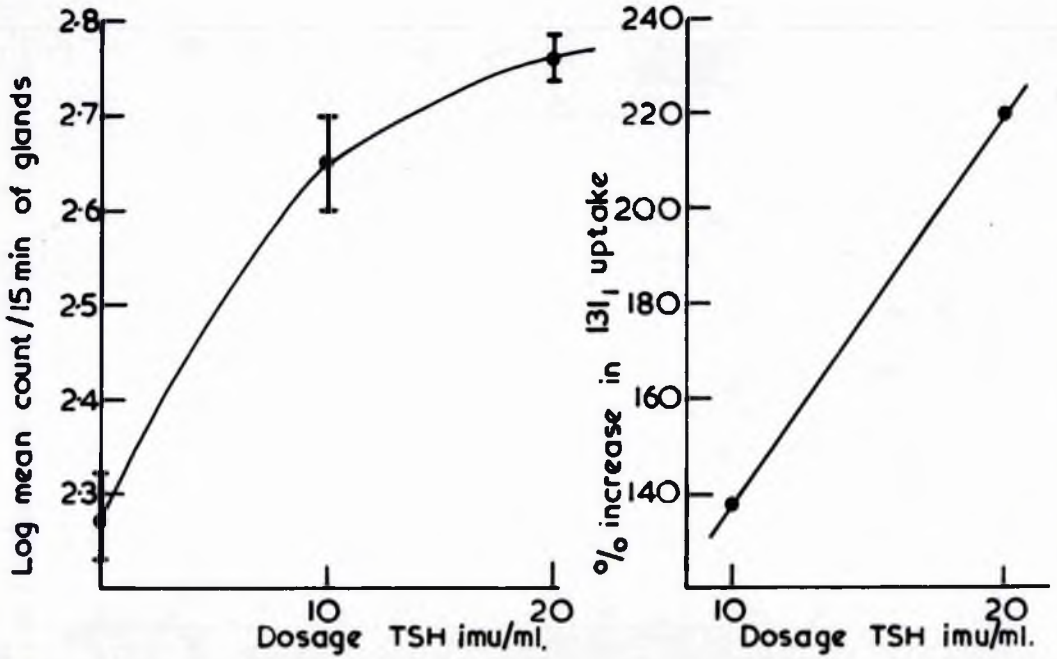
	TSH injected (10 iμ/ml.)	Control
Untreated		
Number of animals	7	4
Mean hind-limb length (mm.)	5.21 (1.2 - 8.1)	6.3 (5.6 - 7.0)
Mean CCPM of glands	5,118	5,792
Log. mean CCPM ± standard deviation	3.71 ±0.67	3.76 ±0.48
Log. mean CCPM ± standard error	3.71 ±0.25	3.76 ±0.24
Pretreated with KI (60γ/1000ml)		
Number of animals	7	7
Mean hind-limb length (mm.)	6.51 (3.8 - 9.1)	5.58 (4.3 - 7.1)
Mean CCPM of glands	1,542	628
Log. mean CCPM ± standard deviation	3.19 ±0.199	2.79 ±0.53
Log. mean CCPM ± standard error	3.19 ±0.075	2.79 ±0.198
% increase over control uptake	14.36%	
Pretreated with thyroxine (1pt./10^9)		
Number of animals	8	5
Mean hind-limb length (mm.)	6.16 (3.3 - 8.2)	5.4 (3.6 - 8.07)
Mean CCPM of glands	7,487	2,742
Log. mean CCPM ± standard deviation	3.87 ±0.37	3.44 ±0.72
Log. mean CCPM ± standard error	3.87 ±0.13	3.44 ±0.32
% increase over control uptake	12.5%	

The results show that variability is somewhat reduced after pretreatment with either thyroxine or potassium iodide (Table XXXIV, Figure 33). Whereas no response was observed as a result of injection of TSH in the group which received no pretreatment, an increase in iodine uptake is apparent in the experimental animals in both pretreatment groups. The response obtained in each case was of the same order although the counting rate in the potassium iodide group was reduced as in the previous experiment using potassium iodide pretreatment. The percentage increase in ^{131}I uptake of the TSH-treated animals over the control animals was 14.4% in the group pretreated with potassium iodide and 12.5% in the group pretreated with thyroxine.

The tadpole has been shown to be extremely sensitive to thyroxine and thyroxine derivatives and has been used as an assay animal for these substances by several workers, (Gaddum, 1927; Dodd & Landgrebe, 1953). Both groups of larvae pretreated with thyroxine showed marked acceleration of metamorphosis. This metamorphic effect makes thyroxine less suitable for use in the pretreatment regime than potassium iodide which has no appreciable effect on metamorphosis.

A series of assays were carried out in an attempt to establish the most satisfactory method of administering the pretreatment and to determine the most effective concentration of potassium iodide.

FIG. 34.


Log mean count/15 min of glands
2·8
2·7
2·6
2·5
2·4
2·3
10
20
Dosage TSH imu/ml.
% increase in 131I uptake
240
220
200
180
160
140
10
20
Dosage TSH imu/ml.

3. The five assays discussed under this heading were designed to determine:-

(a) The most effective concentration at which to carry out pretreatment with potassium iodide.

(b) The most effective method of administration of pretreatment, either prior to or during the injection period.

(c) The minimal concentration of TSH which will induce a measurable increase in iodine uptake by the glands in animals pretreated with potassium iodide.

(i) Routine:-

Number of animals = 36

Starvation period = 7 days

Pretreatment = KI; 100mg/1000 ml; for 48 hrs. prior to injection.

Injection schedule = 0.02 ml./day/5 days.

$^{131}_{I}$ immersion = 10µc/1000 ml.; throughout the injection period.

Results.

TABLE XXXV

	TSH injected (20 imu/ml.)	TSH injected (10 imu/ml.)	Control
Number of animals	9	9	9
Mean hind-limb length (mm.)	3.3 (1.7 - 7.7)	3.9 (2.3 - 6.9)	3.2 (2.1 - 4.2)
Mean count/15 min./ group of 3 animals	584	447	188
range	(661 - 552)	(506 - 394)	(214 - 175)
% increase over control uptake	220%	137%	

The glands were pooled for counting in groups of three animals per planchette and 15 min. counts made on each. The results are recorded in

FIG. 35.

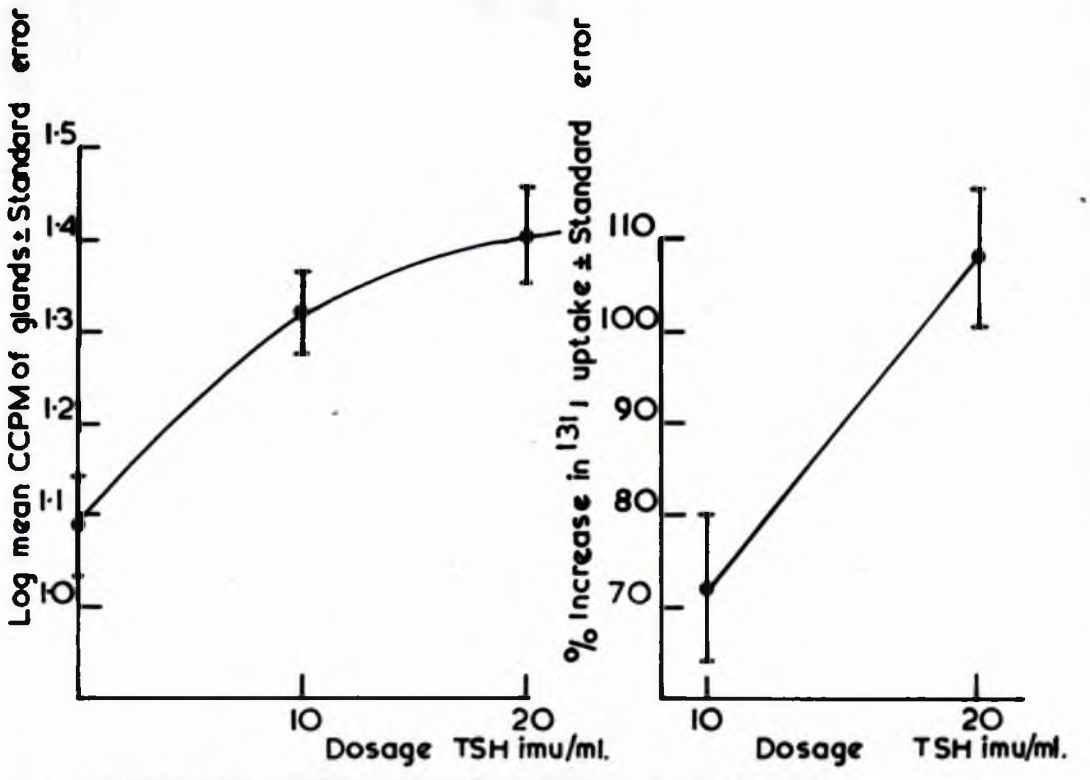

Log mean CCPM of glands ± Standard error
1·5
1·4
1·3
1·2
1·1
1·0
10
20
Dosage TSH imu/ml.
% Increase in ^{131}I uptake ± Standard error
110
100
90
80
70
10
20
Dosage TSH imu/ml.

Table XXXV and Figure 34, expressed in log. mean corrected counts per fifteen minutes and as percentage increase in uptake of ^{131}I of the treated groups over the control value.

(ii) Routine:-

Number of animals	=	30
Starvation period	=	5 days
Pretreatment	=	KI; 100 mg/1000 ml; for 48 hrs. prior to injection.
Injection schedule	=	0.02 ml/day/5 days
^{131}I immersion	=	10µc/1000 ml; throughout the injection period.

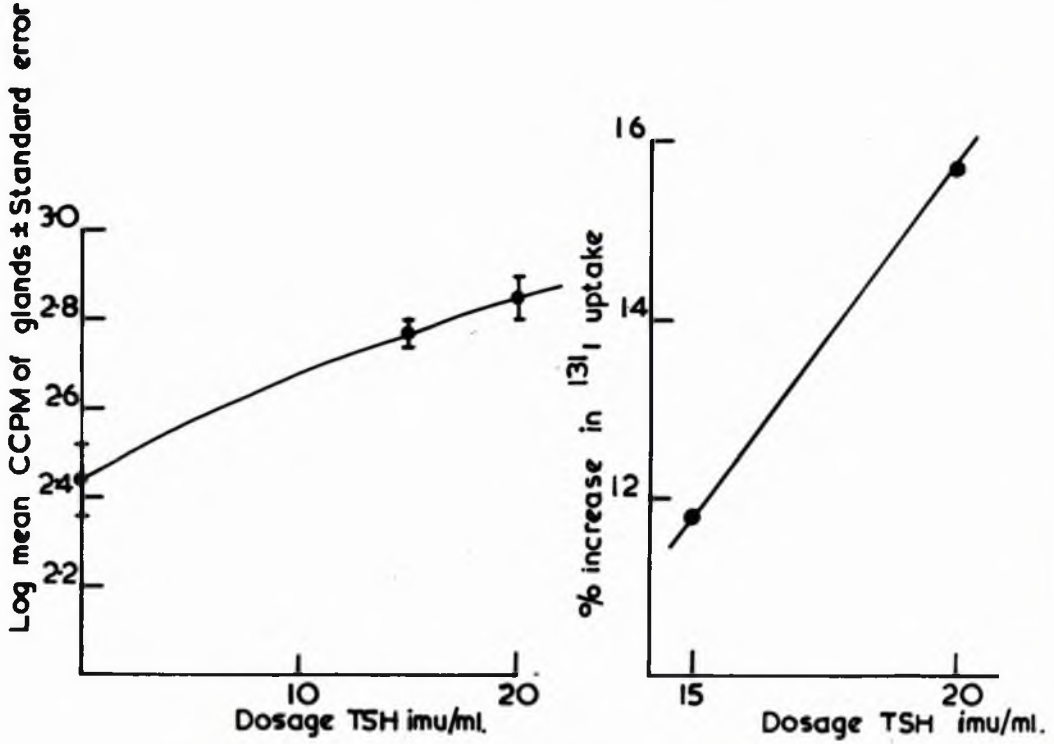
The activity in the glands was determined individually and expressed as corrected counts per minute. Results are recorded in Table XXXVI and Figure 35.

Results.

TABLE XXXVI

	TSH injected (20 imu/ml.)	TSH injected (10 imu/ml.)	Control
Number of animals	10	10	10
Mean hind-limb length (mm.)	4.8 (2.9 - 8.4)	3.9 (2.1 - 6.7)	3.7 (1.4 - 7.3)
Mean CCPM of glands	25.3	21.0	12.2
Log. mean CCPM ± standard deviation	1.40 ±0.139	1.32 ±0.142	1.09 ±0.17
Log. mean CCPM ± standard error	1.40 ±0.044	1.32 ±0.044	1.09 ±0.0
% increase over control uptake	108%	72%	

FIG. 36.


Log mean CCPM of glands ± Standard error
30
28
26
24
22
10
20
Dosage TSH imu/ml.
% increase in ^{131}I uptake
16
14
12
15
20
Dosage TSH imu/ml.

(iii) Routine:-

Number of animals = 30

Starvation period = none

Pretreatment = KI; 100mg/1000ml; 48 hrs. prior to injection.

Injection schedule = 0.02 ml. twice daily for 3 days.

^{131}I immersion = 20µc/1000ml; throughout the injection period.

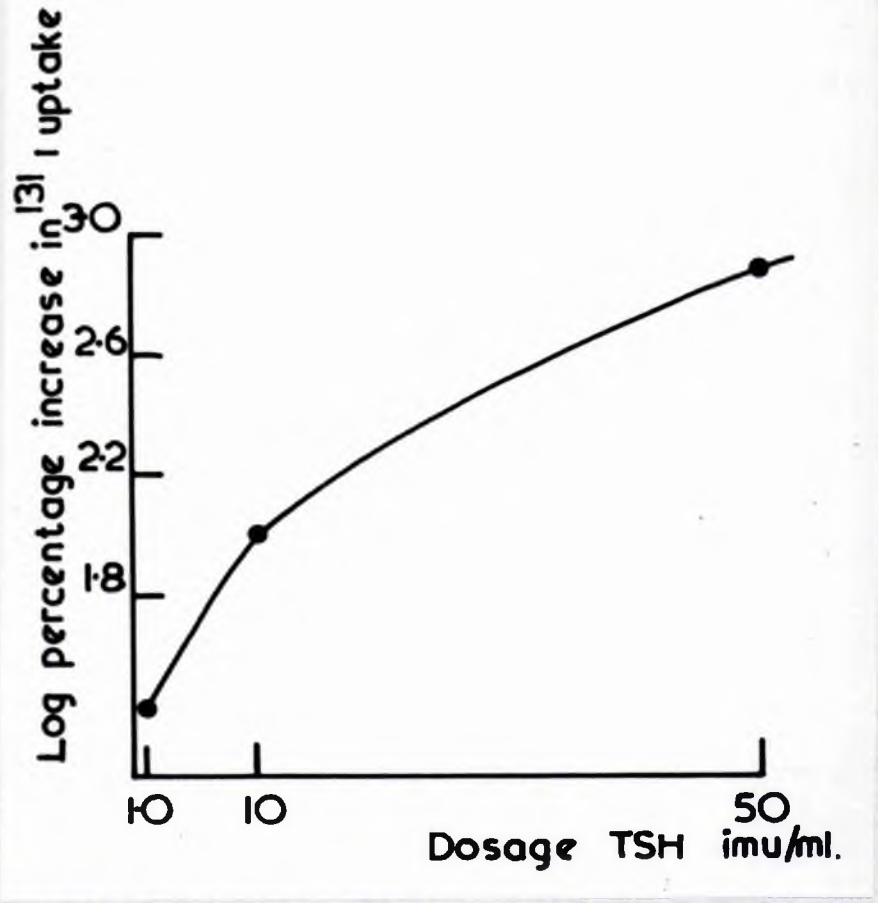
Results.

Injection of TSH was carried out twice daily for three days; the animals were large and healthy and withstood the extra handling well. Results are recorded in Table XXXVII and Figure 36.

TABLE XXXVII

	TSH injected (20 imu/ml.)	TSH injected (15 imu/ml.)	Control
Number of animals	10	9	9
Mean hind-limb length (mm.)	5.9 (3.8 - 8.6)	6.3 (3.9 - 8.2)	4.4 (3.6 - 5.9)
Mean CCPM of glands	707	584	275
Log. mean CCPM ± standard deviation	$2.85^{\pm 0.143}$	$2.77^{\pm 0.091}$	$2.44^{\pm 0.229}$
Log. mean CCPM ± standard error	$2.85^{\pm 0.045}$	$2.77^{\pm 0.03}$	$2.44^{\pm 0.076}$
% increase over control uptake	15.7%	11.8%	

FIG. 37.


Log percentage increase in ^{131}I uptake
3·0
2·6
2·2
1·8
10
10
50
Dosage TSH imu/ml.

(iv) Routine:-

Number of animals = 60

Starvation period = 7 days

Pretreatment = KI; 10µg/1000ml; throughout injection period

Injection schedule = 0.02 ml/day/4 days.

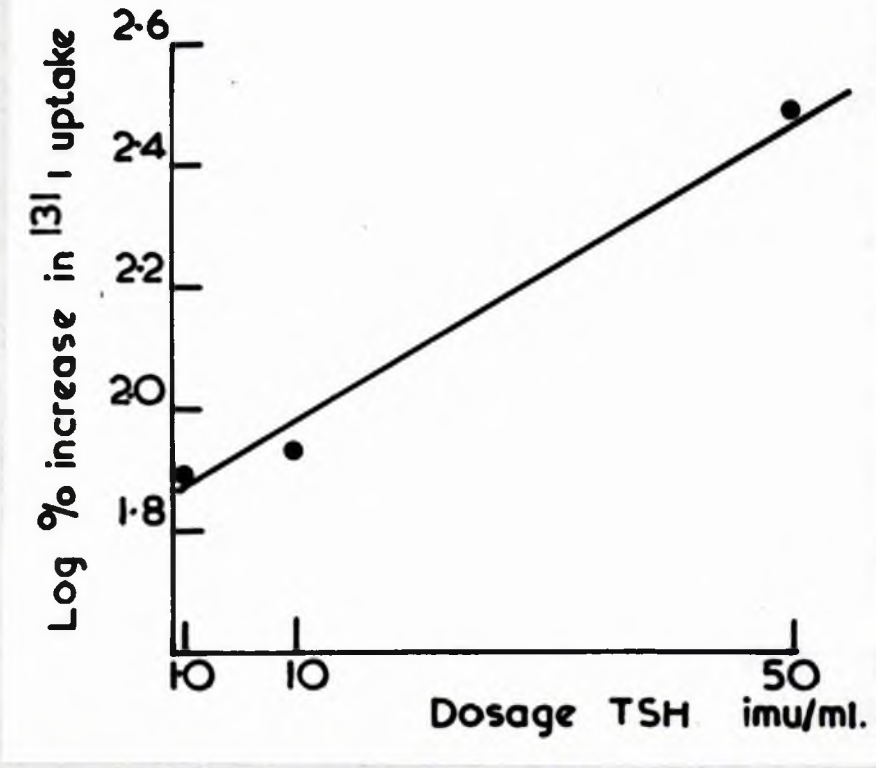
^{131}I immersion = 10µc/1000 ml; throughout the injection period.

Results.

TABLE XXXVIII

	TSH injected (50 imu/ml.)	TSH injected (10 imu/ml.)	TSH injected (1.0 imu/ml.)	Control
Number of animals	10	10	7	7
Mean hind-limb length (mm.)	5.9 (3.8 - 7.9)	4.6 (3.0 - 6.1)	5.3 (3.1 - 8.2)	4.9 (3.4 - 6.4
Mean CCPM of glands	6,725	1,938	1,214	961
Log. mean count ± standard deviation	$3.83^{\pm}0.220$	$3.29^{\pm}0.021$	$3.08^{\pm}0.339$	$2.98^{\pm}0.9$
Log. mean count ± standard error	$3.83^{\pm}0.069$	$3.29^{\pm}0.065$	$3.08^{\pm}0.128$	$2.98^{\pm}0.3$
% increase over control uptake	600%	101.3%	26.3%	

FIG. 38.


Log % increase in ^{131}I uptake
2·6
2·4
2·2
2·0
1·8
10
10
50
Dosage TSH imu/ml.

(v) Routine:-

Number of animals = 40

Starvation period = 5 days

Pretreatment = KI; 10µg/1000 ml; throughout the injection period.

Injection schedule = 0.02 ml/day/5 days.

^{131}I immersion = 10µc/1000 ml; throughout the injection period.

Results.

TABLE XXXIX

	TSH injected (50 imu/ml.)	TSH injected (10 imu/ml.)	TSH injected (1.0 imu/ml.)	Control
Number of animals	10	10	10	9
Mean hind limb-length (mm.)	5.4 (4.1 - 7.2)	5.5 (4.1 - 6.8)	5.7 (3.6 - 7.9)	4.1 (3.4 - 6.6)
Mean CCPM of glands	3,524	2,114	2,030	1,143
Log. mean CCPM ± standard deviation	$3.22^{\pm}0.246$	$3.16^{\pm}0.316$	$3.15^{\pm}0.896$	$3.13^{\pm}0.88$
Log. mean CCPM ± standard error	$3.22^{\pm}0.077$	$3.16^{\pm}0.100$	$3.15^{\pm}0.283$	$3.13^{\pm}0.29$
% increase over control uptake	385%	85%	77.5%	

DISCUSSION.

The conditions prevailing in Experiments (i) and (ii) and Experiments (iv) and (v) are comparable; these may therfore be regarded as duplicates. It was apparent from the preceeding experiments that after pretreatment with iodide an enhanced response to TSH stimulation was obtained (Table XXXIV). The lowest dose of TSH which elicited an increase in ^{131}I uptake was found to be 1.0 imu/ml. A dose-response curve ranging from 1.0 to 50.0 imu/ml. was constructed in Experiments (iv) and (v), (Figures 37 and 38), although the significance of the response to 1.0 imu/ml. is doubtful because of the great variability encountered between responses of individual tadpoles to identical treatment. Not only sensitivity but also discrimination between doses is greatly reduced by this variability. It is however, possible to discriminate between doses of 10 and 20 imu/ml. after pretreatment with potassium iodide.

Three concentrations of potassium iodide, 10µg., 60µg. and 100mg/1000 ml. were used in the course of the investigations, but no marked difference in effect was obtained by increasing or decreasing the concentration. Variability was slightly reduced in each instance and the response to TSH stimulation was enhanced in the pretreated groups.

The mechanism whereby this effect occurs would appear to be one of partial saturation of the gland with non-radioactive iodine. The results suggest that the variability lies in individual differences in the capacity of the glands to accumulate iodine rather than in their ability to respond to TSH. Hence, the response to TSH stimulation is more clearly defined when the basic avidity of the glands for iodine has been satisfied prior to stimulation with TSH. From this it would appear that prolonged pretreatment in a dilute solution of iodide, throughout the starvation period might well be even more effective.

CONCLUSIONS.

The investigations described in this section demonstrate the possibilities and limitations of a method for estimation of TSH dependent on measurement of ^{131}I-uptake by the tadpole thyroid. It can be seen that improved responses to TSH are obtained after starvation and after pre-treatment with iodide. The mechanism by which pretreatment with iodide effects the response to TSH appears to depend on partial saturation of the gland with non-radioactive iodine prior to exposure to ^{131}I. The optimum conditions for obtaining a clearly defined response to administration of TSH at a low order of concentration should therefore be achieved by exposing the test-animals to potassium iodide for a prolonged period prior to injection of TSH.

Before the method can be applied in investigation of serum TSH levels, there remains the problem of further reducing variability between tadpoles so that sensitivity is increased and better discrimination is obtained. In its present form, however, the test could be employed to estimate TSH in more concentrated extracts. So far as concerns simplicity and rapidity, this type of estimation has several advantages over the histometric technique. Thyroidectomy of the assay animals and estimation of the activity present in the glands can be completed within 24 hrs. from the end of the injection period. Further, as compared with the deliberate selection of cells on which to make micrometer measurements, which is necessary in the histometric technique, estimation of TSH stimulation by radiometric criteria is an entirely objective procedure.

PART V

ASSAY OF TSH BY ESTIMATION OF ^{131}I-DISCHARGE

PART V

ASSAY OF TSH BY ESTIMATION OF ^{131}I-DISCHARGE

I. INTRODUCTION.

A number of assay techniques based on measurement of ^{131}I-discharge have been discussed in the review of the literature. It is generally accepted that, while administration of exogenous TSH causes acceleration of both uptake and discharge of iodine by the thyroids, iodine depletion is a more immediate effect than increase in iodine uptake. Keating et al. (1945) found that following administration of TSH, iodine depletion could be measured within the first 24 hrs. whereas increase in iodine uptake became apparent only after 48 hrs. From the point of view of rapidity, therefore, it could be argued that estimation of iodine discharge should be a more useful end-point in assaying TSH than estimation of iodine uptake. Methods of measuring the discharge effect described in the literature include in vivo counting techniques for measuring thyroidal iodine depletion in the chick (Gilliland & Russel-Fraser, 1953; Bates & Cornfield, 1957) and estimation of increase in the blood iodine level in the guineapig (Adams & Purves, 1955) and in the mouse (McKenzie, 1958.).

The possibility of developing an assay method based on estimation of iodine discharge using the Xenopus tadpole was investigated. Estimation of thyroidal iodine uptake is dependent on measurement of the activity present in individual glands. Iodine depletion, on the other hand, could be determined by measuring the activity present in the water in which the animals are immersed. In this way the discharge occurring in a group of animals can be determined by making a single measurement and the need to

thyroidectomise the test-animals does not arise.

In the preceeding studies on the effect of TSH on ^{131}I-uptake by the thyroids, test-animals were selected at a stage of metamorphosis at which the unstimulated ^{131}I-uptake was low and in which the expected response to thyroid stimulation was an increase in the accumulation of iodine. Animals having short hind-limb lengths were therefore used. For the purpose of measuring thyroidal iodine depletion, a high iodine level in the glands of the test - animals is required initially. The unstimulated iodine uptake has been shown to be greater in tadpoles at the peak of metamorphic activity, at the time of eruption of the fore-limbs, than in animals at early limb-bud stages or in which shrinkage and change of shape are occurring. Accordingly, tadpoles of hind-limb length greater than 8 mm. and in which shrinkage had not yet begun to take place were selected as being at a stage at which the response to TSH stimulation would be expected to take the form of accelerated discharge rather than uptake of iodine.

It was first necessary to establish the pattern of iodine discharge in unstimulated tadpoles which had been immersed for a period in a dilute solution of ^{131}I then transferred to water containing no radioactive iodine. Estimation of the iodine level in the water in which the animals are kept is straightforward. After exposure to ^{131}I, the animals are transferred to 500 ml. of ^{131}I-free water from which 9 ml. aliquots are removed for counting in a Veall tube. In order to make valid comparisons between the activity present in succeeding samples, the counting rate is corrected and the results expressed as CCPM/500 ml. of water. Following the same procedure, iodine discharge in response to injection of TSH was then studied under various conditions and the time-relations of the response were investigated.

In the preceding studies on distribution of ^{131}I between thyroid and

other body tissues, it was found that a high proportion of the iodine taken up by animals immersed in a solution of ^{131}I was retained in the extrathyroidal tissues by adsorption. In order to determine what proportion of the iodine taken up during the period of immersion is subsequently discharged, the activity remaining in the experimental jars at the end of the exposure period is compared with the activity in a blank control jar. In studies on discharge in response to administered TSH, the thyroidal iodine content is estimated as well as the increase in iodine discharged into the water to establish that the latter effect is a genuine reflection of thyroidal iodine depletion and not simply release of adsorbed iodine from the extrathyroidal tissues.

II. DISCHARGE OF ^{131}I IN UNTREATED ANIMALS.

1. Serial observations on the iodine discharge were made at increasing time intervals using a group of animals which had previously been immersed in ^{131}I. Five tadpoles were exposed to ^{131}I, at a concentration of 1040/500 ml., for a period of 48 hrs. At the end of this period they were transferred to 500 ml. of clean water containing no radioactive iodine. Aliquots of 9 ml. were withdrawn for counting at increasing time intervals up to 120 hrs. The amount of iodine taken up by the group during the exposure period was estimated as described above and the rate of discharge was then expressed as a percentage of the activity taken up during the immersion period.

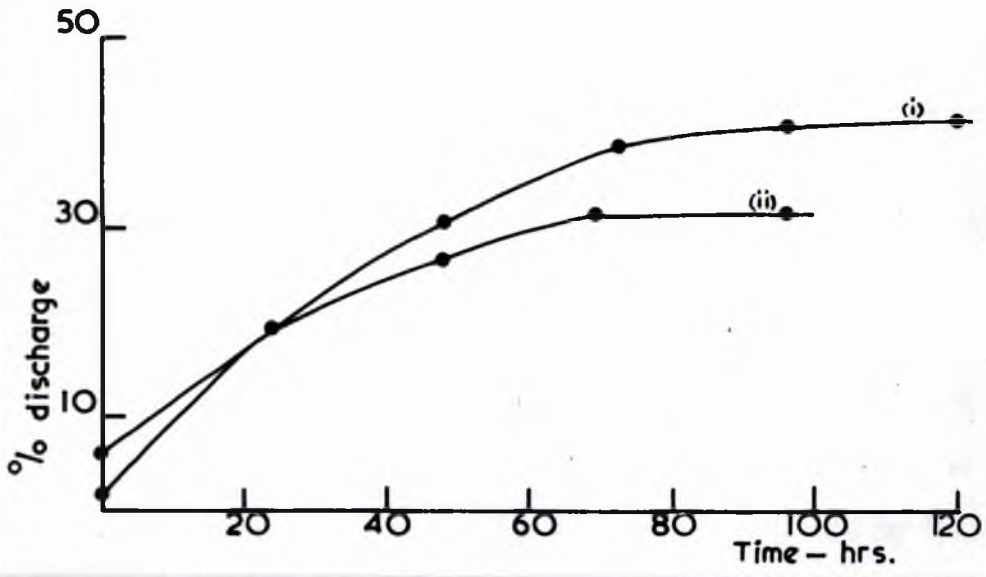
Results.

TABLE XL

	Time	CCPM/500 ml. water	Discharge %
^{131}I uptake:-			
Control 'blank'	after 48 hrs. exposure	311,650	
Experimental	after 48 hrs. exposure	193,900	
Absolute uptake		107,750	
Uptake % =	37.8%		
^{131}I discharge:-	15 min.	1,650	1.4
	24 hrs.	22,750	19.3
	48 hrs.	36,200	30.7
	72 hrs.	45,250	38.4
	96 hrs.	47,750	40.5
	120 hrs.	48,500	41.0

FIG. 39.

Discharge of ^{131}I in untreated animals. Serial observations on 2 groups of five



2. The procedure described in (1) was repeated using a group of 8 animals.

Results.

TABLE XLI

	Time	CCPM/500 ml.	Discharge %
^{131}I uptake:-			
Control 'blank'	after 48 hrs. exposure.	210,100	
	after 48 hrs. exposure.	139,900	
Absolute uptake		70,200	
Uptake % =	33.4%		
^{131}I discharge:-	15 min.	950	5.86
	24 hrs.	3,150	19.45
	48 hrs.	4,350	26.8
	69 hrs.	5,050	31.2
	96 hrs.	5,050	31.2

From the results recorded above (Tables XL and XLI) it can be seen that when a group of animals previously exposed to ^{131}I is transferred into clean water some of the iodine originally taken up is discharged over a period of 72 hrs., up to 30 - 40% of the original uptake (Figure 39). It appears that after 72 hrs. a state of equilibrium is reached between the level of iodine retained in the tadpole and the amount discharged into the water; thus no further discharge occurs. In a preceeding section it was demonstrated that a relatively large amount of iodine is retained in the body of the tadpole by a process of adsorption and that the absolute amount of iodine present in the extrathyroidal tissues is considerably greater than that held in the glands. It is probable that some of this unbound

iodine is "washed out" when the animal is transferred to clean water and that the discharged iodine is not entirely thyroidal in origin.

FIG. 40.

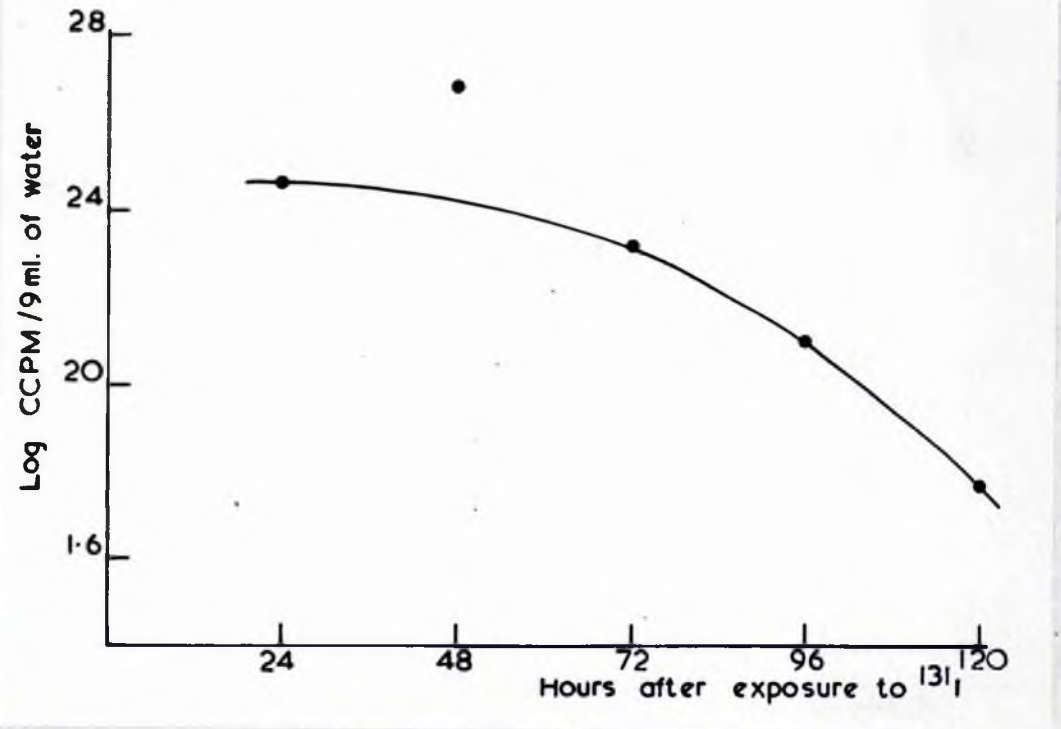


Fig. 40

Discharge of ^{131}I in untreated larvae. Serial observations on a single group of animals, changing the water every 24 hrs.

3. The two preceeding experiments established that in 72 hrs., a state of equilibrium is reached between iodine discharged into the water and iodine retained in the extrathyroidal tissues. Experiments were next carried out to determine whether, by changing the water between observations, it is possible to accelerate the process of equilibration and, at the same time, to cause increased release of unbound iodine.

(i) Ten animals were exposed to ^{131}I, (10μc/500 ml.) for 48 hrs. At the end of this period they were transferred to 500 ml. clean water and samples taken for counting 15 min. and 24 hrs. after the change. The water was then changed at 24 hr. intervals for a period of 120 hrs. and samples taken at 15 min. and 24 hrs. after each change.

Results.

T A B L E XLII

	Time	CCPM/500 ml.	Discharge %
^{131}I uptake:-			
Control 'blank'	after 48 hrs. exposure	356,400	
Experimental	after 48 hrs. exposure	267,350	
Absolute uptake		89,050	
Uptake % =	25%		
^{131}I discharge:-	15 min.	2,800	
	24 hrs.	14,300	16.2
	24 hrs. + 15 min.	600	
	48 hrs.	24,150	27.2
	48 hrs. + 15 min.	900	
	72 hrs.	10,450	11.75
	72 hrs. + 15 min.	1,850	
	96 hrs.	6,350	7.15
	96 hrs. + 15 min.	2,300	
	120 hrs.	2,950	3.32
Total amount discharged			65.62%

FIG. 41.

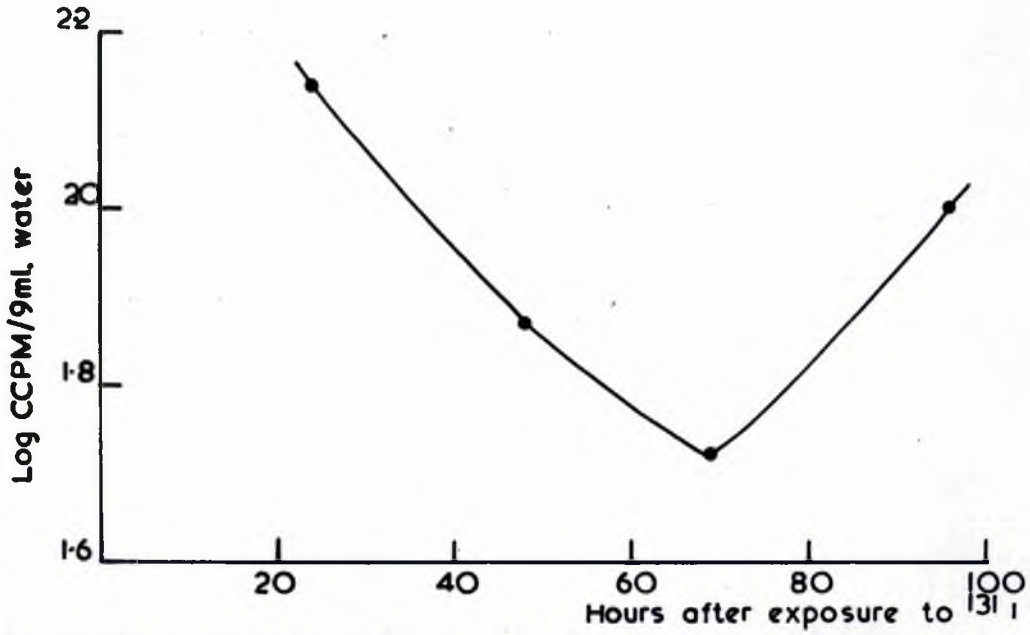


Fig. 41

Discharge of ^{131}I in untreated larvae. Observations on a single group of animals, changing the water every 24 hrs.

(ii) The above experiment was repeated using a group of 8 animals exposed to ^{131}I, (10μc/500 ml.) for 48 hrs. the water being changed at 24 hr. intervals over a period of 96 hrs.

Results.

TABLE XLIII

	Time	CCPM/500 ml.	Discharge %
^{131}I-uptake:-			
Control 'blank'	after 48 hrs. exposure	210,100	
	after 48 hrs. exposure	165,150	
Absolute uptake		44,950	
Uptake % =	21.4%		
^{131}I-discharge:-	15 min.	1,200	
	24 hrs.	6,850	15.6
	24 hrs. + 15 min.	900	
	48 hrs.	3,700	8.25
	48 hrs. + 15 min.	550	
	69 hrs.	2,600	5.8
	69 hrs. + 15 min.	650	
	96 hrs.	5,000	11.3
Total amount discharged			40.95%

The results obtained (Tables XLII and XLIII) confirm the suggestion that it is possible to cause the unbound iodine contained in the extrathyroidal tissues to be discharged into the water by subjecting the animals to a series of changes of water. The amount of iodine discharged during 120 hrs. is increased from 41% in the group which had only one change of water to 65.6% in the group where the water was changed 5 times. It appears, therefore, that by changing the water at intervals for a sufficient length of time it would be

possible to wash out most of the unbound iodine from the extrathyroidal tissues.

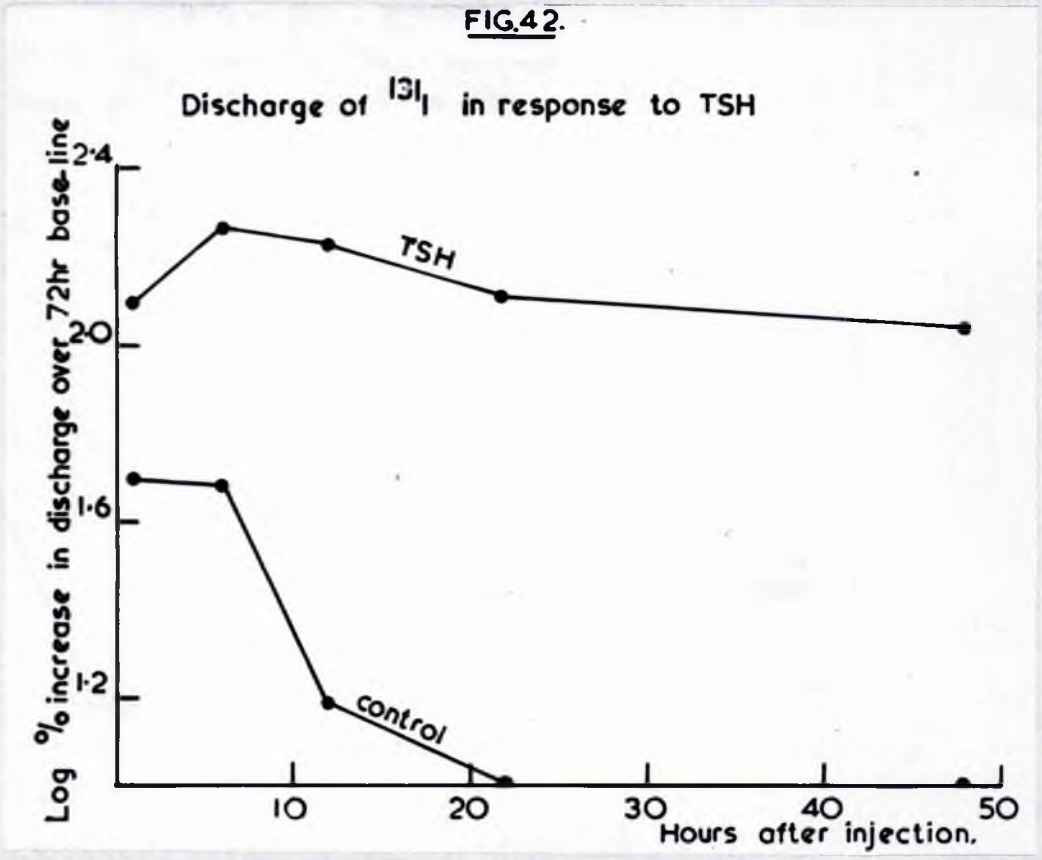
The preceeding experiments demonstrate that in untreated animals exposed to ^{131}I then transferred to ^{131}I free water, discharge of adsorbed iodine occurs until a state of equilibrium is reached at 72 hrs. after the change. When the animals are transferred from the ^{131}I solution to ^{131}I free water which is changed at intervals, a greater proportion of the iodine taken up is discharged. Estimation of increase in discharge of ^{131}I into the water in response to administered TSH may be regarded as an indirect method of measurement. This being the case, since the extrathyroidal tissues in the tadpole retain relatively large amounts of ^{131}I it would be more satisfactory to remove this prior to administration of the test substances. To achieve this, by a series of changes of the water in which the animals are immersed, however, appears to require more than 120 hrs. Less time, 72 hrs., is required to establish a state of equilibrium between the water and extrathyroidal tissues when only one change of water is made. Experiments were therefore carried out to determine whether a further increase in ^{131}I discharge occurred in response to an injection of TSH in animals which had been equilibrated for 72 hrs.

III. DISCHARGE OF ^{131}I IN RESPONSE TO TSH STIMULATION.

1. The effect of a single injection of TSH on discharge of ^{131}I after 72 hrs. equilibrium in ^{131}I-free water.

(i) Groups of 5 animals were exposed to ^{131}I, (10μc/500 ml.) for 48 hrs., then transferred to clean water in which they were allowed to equilibrate for 72 hrs. At the end of this time an aliquot was removed to establish a base line for the concentration of ^{131}I in the water prior to injection. A single large dose of TSH was given (0.4 imu/0.01 ml.) to the experimental group and 0.01 ml. of saline to the control group. Samples of 5 ml. were removed at intervals up to 48 hrs. and made up to 9 ml. for counting. The results were corrected to CCPM/500 ml. and expressed as the percentage increase over the basic iodine level prior to injection.

FIG.42.


Discharge of ^{131}I in response to TSH
Log % increase in discharge over 72hr base-line
2·4
2·0
1·6
1·2
TSH
control
10
20
30
40
50
Hours after injection.

Results.

T A B L E XLIV

^{131}I uptake prior to treatment

	Time	CCPM/500 ml.	Absolute Uptake	Uptake %
Control 'blank'	after 48 hrs. exposure	291,600		
Experimental group	after 48 hrs. exposure	59,200	232,400	80
Control group	after 48 hrs. exposure	70,500	221,100	75.5

^{131}I discharge:-

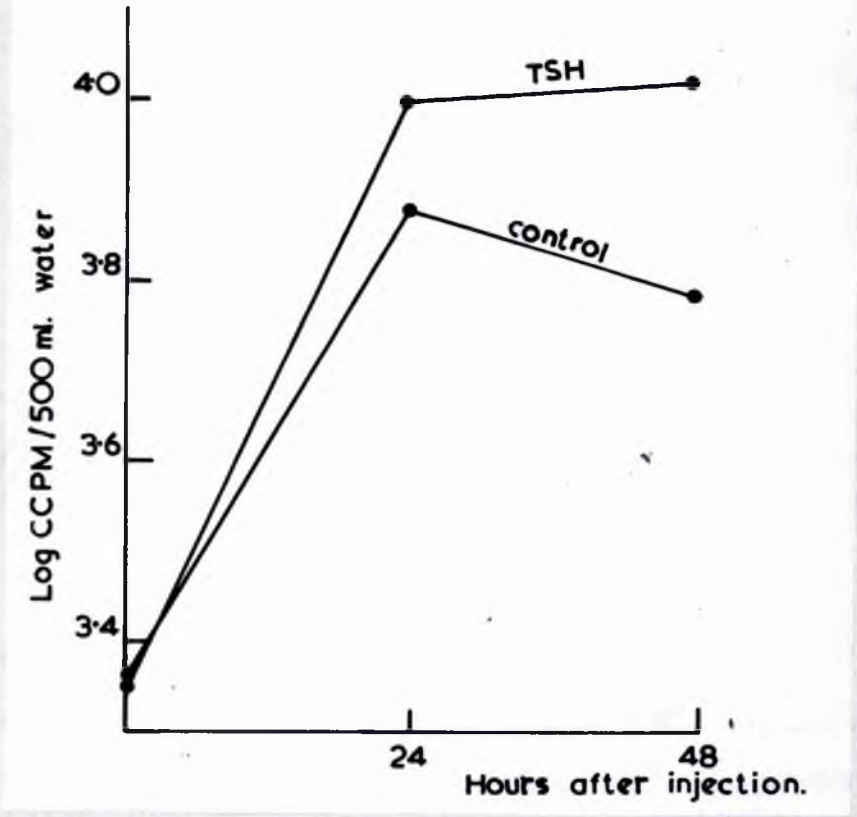
Treatment		Time after injection (hrs.)					
		0	1	6	12	24	48
TSH injected (0.4 imu)	Actual count (CCPM/500 ml)	25,219	56,719	71,797	68,095	57,393	47,379
	Absolute increase		31,500	46,578	42,876	32,174	22,160
	% increase		125%	185%	170%	128%	88%
Saline injected control	Actual count (CCPM/500 ml)	35,461	52,729	52,127	40,997	31,207	32,053
	Absolute increase		17,268	16,666	5,536	-	-
	% increase		48.7%	46.7%	15.6%		

It can be seen (Table XLIV, Figure 42) that a marked rise in the rate of discharge of iodine occurs 6 hrs. after a single large dose of TSH is given. The slight discharge encountered in the saline-injected control group is probably partly due to handling and injection. The amount of iodine still retained at the end of the experiment can be calculated from the results given above. It is found that the TSH treated group retained 78.2% of the original amount taken up and the saline injected group retained

84.2%. The amount of iodine discharged into the water at 48 hrs. after injection is 20.5% of the original uptake in the TSH-treated group and 14.5% in the saline-injected group. Thus 98.7% of the original uptake is accounted for in each case. This figure indicates that the error introduced by the process of pipetting off serial samples, and transferring the animals from jar to jar is of small significance.

FIG. 43.

Effect of a single injection of TSH on ^{131}I discharge



(ii) The procedure described in the previous experiment was followed using a smaller dose of TSH, (10 imu/ml.). The animals were exposed to iodine, allowed to equilibrate for 72 hrs. prior to injection of 0.01 ml. of the test substance and samples were removed for counting at 24 and 48 hrs. after injection.

Results.

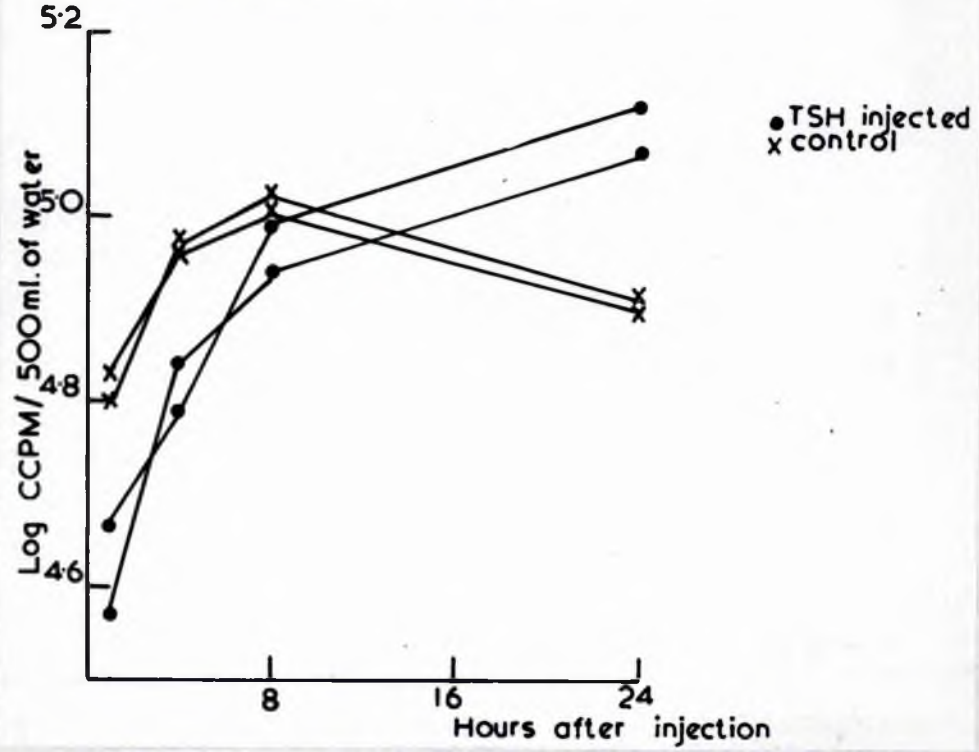
TABLE XLV

	Equilibrated at 72 hrs.	Time after injection 24 hrs.	48 hrs.
TSH treated:-			
Actual count (CCPM/500 ml.)	2,229	9,572	11,789
Absolute increase		7,243	9,560
% increase		326%	430%
Saline injected control:-			
Actual count (CCPM/500 ml.)	2,229	7,599	5,986
Absolute increase		5,370	3,757
% increase		240%	169%

In this experiment it has again been possible to demonstrate a response to TSH. (Table XLV, Figure 43). It appears that with the lower dose-level of TSH the response occurs more slowly than when a massive dose is administered. The difference in discharge between the treated group and the control group is greater at 48 hrs. than at 24 hrs. in animals given 0.1 imu of TSH. In the previous experiment in which the amount of TSH administered was 0.4 imu, the maximum response was demonstrated at 6 hrs. after injection.

FIG. 44.

Detail of time relationship of response to TSH



(iii) In this experiment, the discharge of ^{131}I which results from TSH treatment was followed in greater detail. Twenty animals were starved for 7 days and immersed in ^{131}I (10μc/1000 ml) for the last 48 hrs. of the starvation period. They were then transferred to clean water in groups of 5 per 500 ml. and allowed to equilibrate for one hour prior to injection. Two groups were given 0.02 ml. of TSH (20 imu/ml.) and the two control groups 0.02 ml. of saline. Samples were removed for counting at intervals up to 24 hrs. and the pattern of ^{131}I discharge determined (Table XLVIa). At the end of this period, the animals were thyroidectomised and the activity remaining in the glands determined (Table XLVIb).

Results.

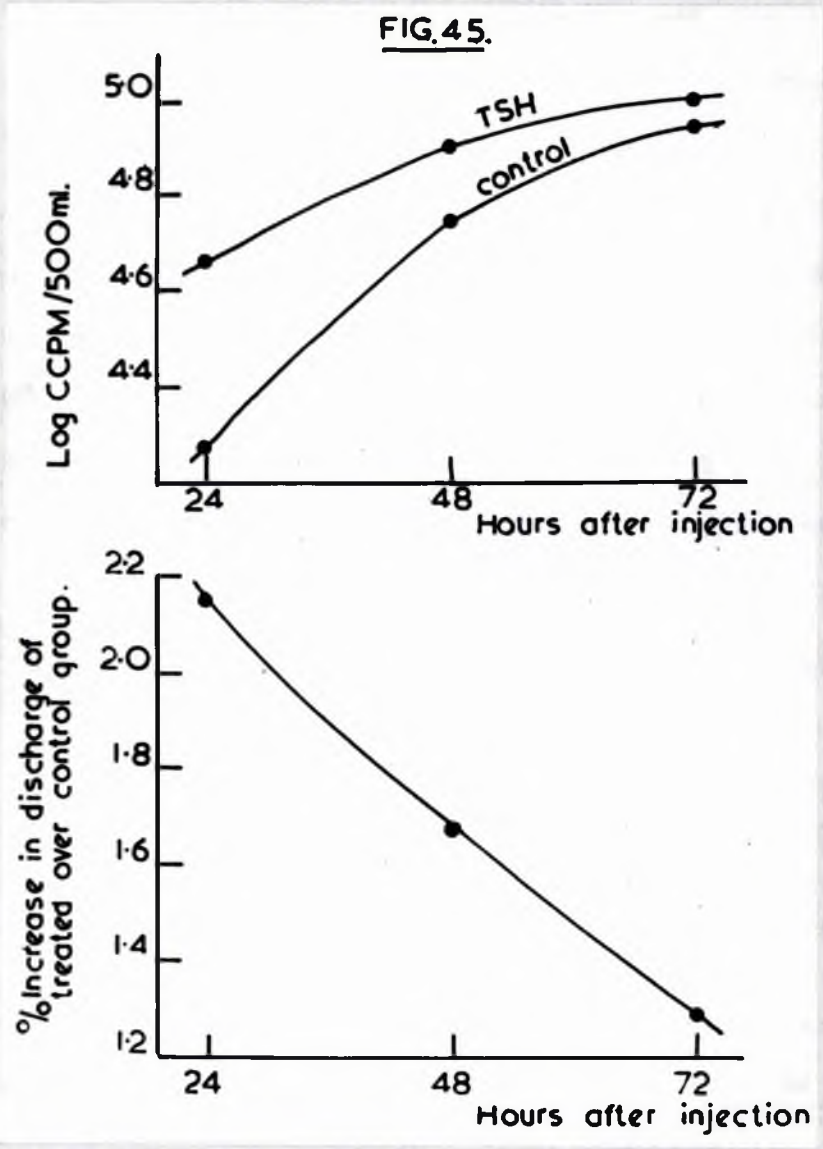
TABLE XLVI

		TSH 20 imu/ml.	TSH 20 imu/ml.	Control	Control
a) Discharge effect of injected TSH:-					
CCPM/500 ml. water	0 hrs.	850	350	1,700	1,100
	1 hr.	46,077	37,247	63,244	67,152
	4 hrs.	62,031	69,723	92,803	90,559
	8 hrs.	98,940	86,236	105,166	100,919
	24 hrs.	131,550	118,169	81,999	78,752
% increase in discharge	0 - 24 hrs.	15,420%	33,360%	4,725%	7,050%
b) Thyroidal ^{131}I content:-					
Mean CCPM of glands		1,257	2,383	1,480	3,540
Log. mean count ± standard deviation		$3.09^{\pm 0.493}$	$3.38^{\pm 0.442}$	$3.17^{\pm 0.271}$	$3.55^{\pm 0.421}$
Log. mean count ± standard error		$3.09^{\pm 0.221}$	$3.38^{\pm 0.221}$	$3.17^{\pm 0.121}$	$3.55^{\pm 0.188}$

As in previous experiments, a difference in the amount of iodine discharged by the control groups and the TSH treated groups was apparent at 24 hrs. (Table XLVIa, Figure 44). The duplicate control groups and treated groups gave parallel results. All four groups showed the initial rise in discharge attributed to mechanical effects of the injected procedure. It appears from these findings that a response to TSH can be demonstrated without allowing 72 hrs. for equilibration in clean water.

It was not possible to show any significant difference in the iodine content of the glands between the TSH treated groups and the saline-treated groups. The apparent discharge effect of administered TSH, as measured indirectly by estimating the activity present in the water, was not, in this instance, mirrored in a lowered iodine content in the glands of the treated groups. This may be attributed partly to individual variability between the test-animals.

FIG. 45.


5·0
4·8
4·6
4·4
Log CCPM/500ml.
TSH
control
24
48
72
Hours after injection
2·2
2·0
1·8
1·6
1·4
1·2
% Increase in discharge of treated over control group.
24
48
72
Hours after injection

(iv) Two experiments were carried out in which discharge of ^{131}I in response to multiple injections of TSH was followed over a period of three days.

a) Two groups of 7 animals each were exposed to ^{131}I (20µc/1000 ml.) for 48 hrs. and then transferred to 500 ml. of clean water. TSH was administered at a concentration of 10 imu/ml., six injections being given over a period of 3 days. It was possible to increase the total amount of TSH administered by injecting 0.03 ml. because the animals used were large. Water samples were removed for counting at 24 hr. intervals in order to determine the absolute rate of discharge in both the treated and the control group; percentage increase in discharge by the treated group over the control group was calculated (Table XLVIIa, Figure 45). The activity remaining in the glands at the end of the injection period was also determined as in the previous experiment (Table XLVIIb).

Results.

TABLE XLVII

a) Discharge effect of injected TSH:-

CCPM/500 ml. of water

Time after first injection (hrs.)	24	48	72
TSH injected (100imu/ml.)	45,360	79,997	103,429
Saline injected control	18,750	54,605	86,627
Absolute increase	26,610	25,392	16,802
% increase	142%	47%	19.4%

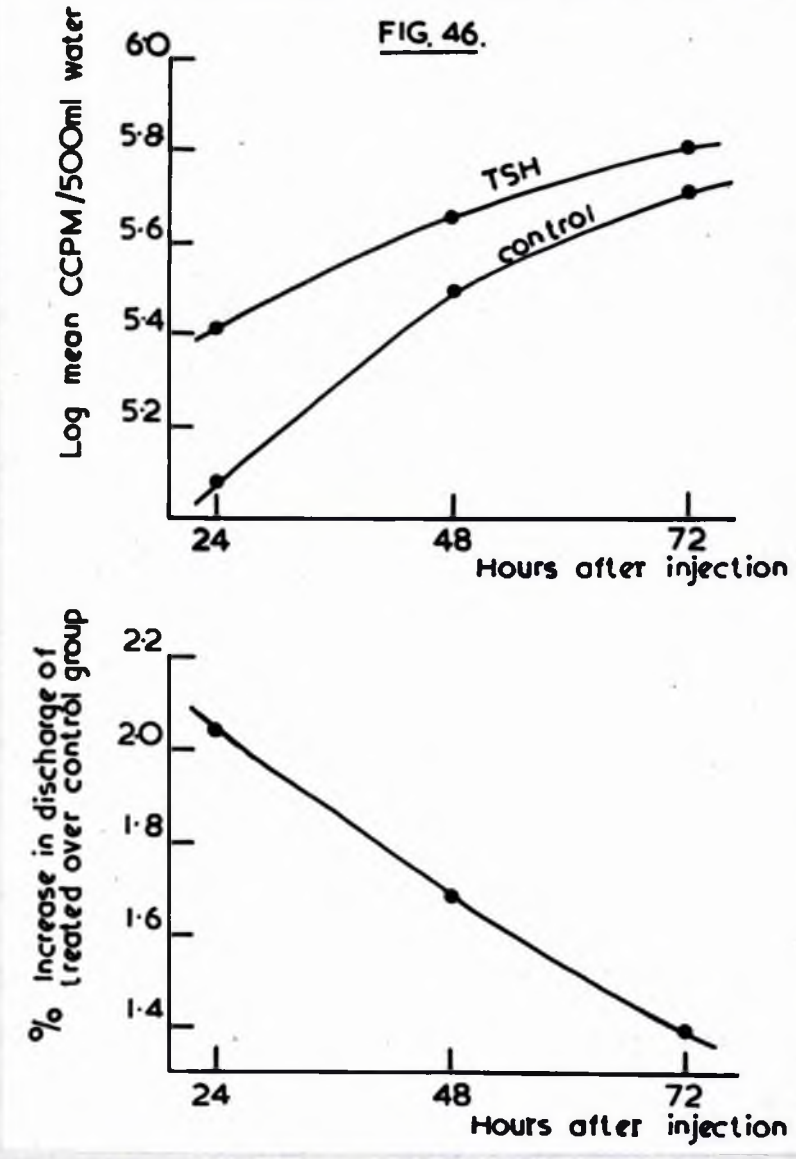
b) Thyroidal ^{131}I content:-

	TSH injected (100 imu/ml.)	Control
Number of animals	7	6
Mean hind-limb length (mm.)	12.25(9.8-14.3)	10.9(9.3-13.1)
Mean CCPM of glands	24,393	35,497
Log. mean count ± standard deviation	4.39 ±0.432	4.55 ±0.301
Log. mean count ± standard error	4.39 ±0.163	4.55 ±0.123

Twenty-four hours after the first injection, discharge by the TSH treated group was found to be 14.2% higher than in the control group. Discharge in both groups continued throughout the 72 hr. injection period, but was only 19.4% higher in the TSH-treated group than in the control group at the end of this time. (Table XLVIIa. Figure 45). It appears, therefore, that discharge of ^{131}I in response to administered TSH is not increased as a result of giving multiple injections.

On the other hand, it was found that, as a result of giving multiple injections, the mean value for the amount of ^{131}I remaining in the glands at the end of the injection period was lower in the TSH treated group than in the control group, although variability was considerable (Table XLVIIb.).

FIG. 46.


Log mean CCPM/500ml water
6·0
5·8
5·6
5·4
5·2
TSH
control
24
48
72
Hours after injection
% Increase in discharge of treated over control group
2·2
2·0
1·8
1·6
1·4
24
48
72
Hours after injection

b) The procedure details above was followed in a duplicate experiment using groups of 7 animals and giving multiple injections of TSH at a concentration of 50 imu/ml.

Results.

TABLE XLVIII

a) Discharge effect of TSH:-

CCPM/500 ml. of water.

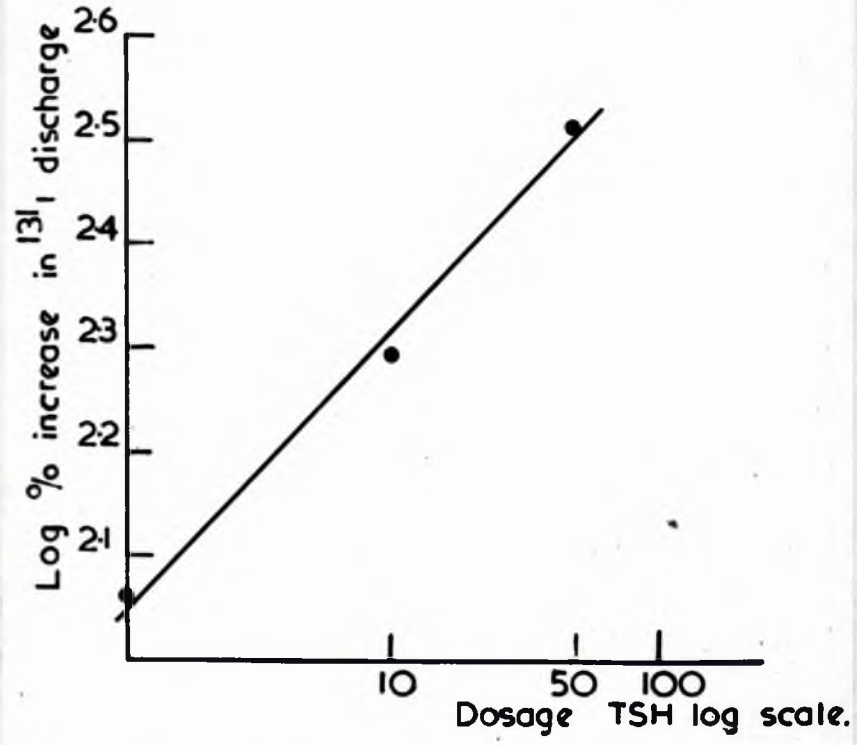
Time after first injection (hrs.)	24	48	72
TSH injected (50 imu/ml.)	255,350	450,283	630,931
Saline injected control	120,950	305,680	506,176
Absolute increase	134,400	144,603	124,755
% increase	110.5%	47.5%	24.7%

b) Thyroidal ^{131}I content:-

	TSH injected (50 imu/ml.)	Control
Number of animals	6	7
Mean hind-limb length (mm.)	11.6 (9.8 - 14.3)	13.4 (9.3 - 16.7)
Mean CCPM of glands	18,511	16,010
Log. mean count ± standard deviation	$4.27^{\pm 0.495}$	$4.20^{\pm 0.279}$
Log. mean count ± standard error	$4.27^{\pm 0.202}$	$4.20^{\pm 0.114}$

These results (Table XLVIIIa, Figure 46) show the same response pattern as was demonstrated in the previous experiment. The percentage increase in discharge of the TSH-injected group over the control group dropped from 110.5% at 24 hrs. to 27.7% at 72 hrs. This would seem to confirm that multiple injections do not improve the response to TSH when measured by discharge of ^{131}I. There was found to be no significant different in the amount of iodine retained by the glands of the two groups at the end of the injection period (Table XLVIIIb).

FIG. 47.


Log % increase in ^{131}I discharge
2·6
2·5
2·4
2·3
2·2
2·1
10
50
100
Dosage TSH log scale.

(v) Response to injection of TSH at two dose-levels.

30 animals were starved for 7 days prior to injection and immersed in ^{131}I (20μc/1000 ml.) for the last 24 hrs. of the starvation period. Having demonstrated in the two preceding experiments that multiple injections, given throughout the period during which discharge estimations were made, appeared to have no influence towards increasing the response obtained, two injections of 0.02 ml. were given with an intervale of 6 hrs. between and the response was estimated 24 hrs. after the first injection. Prior to injection the animals were allowed to equilibrate in clean water for 1 hr. and a sample withdrawn for counting to obtain a base-line on which to calculate percentage increase in discharge at 24 hrs. At the end of the 24 hr. period during which the discharge estimation was carried out, the test-animals were thyroidectomised and the ^{131}I content of the glands determined.

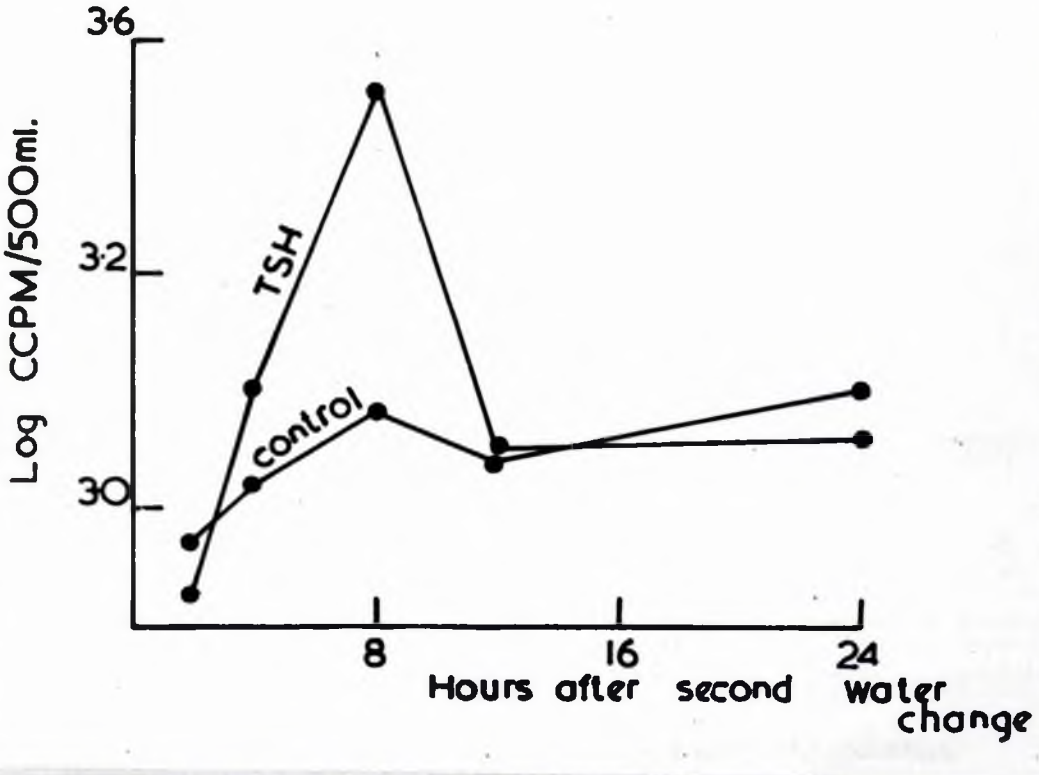
Results.

TABLE XLIX

	TSH injected (50 imu/ml.)	TSH injected (10 imu/ml.)	Control
a) Discharge effect of TSH:-			
CCPM/500 ml. water 1 hr.	170,250	281,950	269,800
CCPM/500 ml. water 24 hrs.	622,752	828,006	578,598
Absolute increase	552,502	546,056	308,798
% increase	328%	194.5%	45%
b) Thyroidal ^{131}I content:-			
Number of animals	8	8	9
Mean hind-limb length (mm.)	8.6 (7.1-11.4)	9.8 (7.3-12.7)	9.4 (4.5-12.9)
Mean CCPM of glands	5,688.0	11,438.0	9,447.0
Log. mean count ± standard deviation	$3.76^{\pm}0.249$	$4.06^{\pm}0.422$	$3.98^{\pm}0.568$
Log. mean count ± standard error	$3.76^{\pm}0.088$	$4.06^{\pm}0.149$	$3.98^{\pm}0.189$

A quantitative dose-response curve was obtained to 10 and 50 ims/ml. of TSH (Table XLIXa, Figure 47.) by this method, but it is clear that the sensitivity is reduced because unbound iodine, present in the extrathyroidal tissues, diffuses into the water quite apart from any iodine actively discharged in response to stimulation by TSH.

FIG. 48.


3·6
3·2
3·0
Log CCPM/500ml.
TSH
control
8
16
24
Hours after second water change

(vi) It was found in experiment (iii) (Figure 44) that discharge of iodine occurred initially to the same extent in both control and experimental groups due to mechanical effects. The response to TSH only became apparent after 16 hrs. If, therefore, the water in which the animals are kept is changed at 16 hrs. after injection, when this effect is complete, the response to TSH should be more clearly defined at 24 hrs.

To determine whether, in fact, an improved response is obtained under these circumstances, two groups of five animals were exposed to ^{131}I, (20μc/1000 ml.) for 48 hrs. The water was then changed and two injections of 0.02 ml. of TSH at a concentration of 10 imu/ml. were given with an intervale of 6 hrs. between. Sixteen hours after the first injection the water was changed for the second time; samples were then removed for counting at intervals up to 24 hrs.

Results.

T A B L E L

Time after second change of water (hrs.)	2	4	8	12	24
CCPM/500 ml. water					
TSH injected (20 imu/ml.)	450	1,038	3,342	757	1,033
Saline injected control.	550	697	937	796	842
Absolute increase	-	341	2,405	-	-
% increase			256.5%		

It was found that the peak of discharge in the TSH-treated group occurred 8 hrs. after the water was changed for the second time. (Table L, Figure 48). The response also appears to be more clearly defined than in previous assays.

IV. DISCUSSION AND CONCLUSIONS

Before proceeding to investigate the effect of TSH on ^{131}I-discharge in tadpoles previously immersed in a dilute solution of iodine, the pattern of discharge of ^{131}I in unstimulated larvae was first studied. In untreated animals exposed to ^{131}I then transferred to clean water, the amount of ^{131}I discharged was found to increase gradually over a period of 72 hrs. At the end of this time a state of equilibrium was established and no further discharge occurred. When the animals were exposed to a series of changes of water at 24 hr. intervals, the iodine present in the water at the end of each 24 hr. period gradually decreased, but the percentage of the original uptake accounted for during the course of the discharge period was found to be greater after a series of changes of water than after only one change. It was concluded, from these findings, that iodine retained in the extrathyroidal tissues is "washed out", as a result of placing the animals in ^{131}I-free water, until a state of equilibrium is established between iodine retained in the tissues and iodine released into the water. By submitting the animals to a series of changes of ^{131}I-free water, a greater proportion of the extrathyroidal iodine was released than when only one change was employed.

Response to TSH injection was investigated under various conditions. In animals in which a state of equilibrium had been established, the maximum discharge of ^{131}I was found to occur 6 hrs. after a single injection of TSH at a high concentration and between 24 and 48 hrs. after injection of TSH at a lower dose-level. This was interpreted as indicating a slower response-time when TSH was administered at the lower dose-level. When multiple injections of TSH were given, the maximum response was still found to occur 24 hrs. after the first injection; succeeding injections appeared to have no effect on the rate of ^{131}I-discharge.

When an assay was performed, an initial discharge effect was observed in both the TSH-treated group and the saline-treated control. This effect was considered to be mechanical, as a result of injection and handling the animals.

A more clearly defined response to TSH was obtained when the test-animals were submitted to a second change of water, 16 hrs. after injection, at a time when the "mechanical" effect was complete. Discharge of ^{131}I in response to TSH was then found to reach a peak 8 hrs. after the second change of water.

The activity present in the glands at the end of a discharge determination was not always found to mirror the effect of TSH as indicated by the amount of iodine discharged into the water. Because the animals used in these investigations had hind-limb lengths between 8 and 12 mm., and were at the stage of maximum iodine uptake, the ^{131}I remaining in the glands was high and variability between individuals was considerable. The failure to obtain a measurable discharge effect by estimating the thyroidal ^{131}I content may be attributed partly to this high degree of variability.

The optimum conditions under which to obtain a measurable response of ^{131}I discharge after administration of TSH would prevail when the extra-thyroidal iodine level is low. It appears that, to achieve such conditions, a prolonged series of changes of water would be necessary. It was found that ^{131}I discharge continued, without TSH stimulation, for as long as 120 hrs. after removing the animals from the ^{131}I solution, when the clean water in which they were placed was changed at 24 hr. intervals. This being the case, the time required to complete the assay would be considerable.

Two alternative routines for estimating TSH by discharge of radioactive iodine are suggested:-

1. Estimation of discharge in animals in which a low level of extrathyroidal iodine has been induced by serial washing in ^{131}I-free water.

Although, clearly, a more accurate estimation of TSH would be obtained under these conditions than when the animals used have a high level of extrathyroidal iodine, this routine was discarded as being impractical from the point of view of the time required to prepare the animals prior to administration of the test-substance.

2. Estimation of discharge in animals in which a state of equilibrium between extrathyroidal iodine and iodine discharged into the water has been established in a preliminary equilibration period.

Using animals prepared in this way, a response was obtained to administration of TSH at a concentration of 10 imu/ml. The optimum response time at this dose-level was found to be 24 hrs. Prior to this time, an initial increase in the amount of iodine released occurred in both the experimental group and the control group, which was thought to be due to the mechanical disturbance resulting from the injection procedure.

In the experiments carried out following the equilibration routine, the time allowed to establish equilibrium was varied from 72 hrs., at which time the unstimulated release of ^{131}I has been shown to be complete, to 1 hr. Equilibrium is not established after one hour in ^{131}I-free water, but sufficient extrathyroidal iodine has been released into the water to be used as a "base-line" against which the increase in ^{131}I-discharge in response to TSH stimulation can be measured. Using the shorter equilibration period and changing the water for a second time 16 hrs. after injection of TSH, it was possible to obtain a response to 10 imu/ml. more clearly defined than in experiments in which the water had not been changed

twice. The level of ^{131}I-discharge by the saline treated group remained low and steady under these conditions. The iodine level in the TSH-treated group increased abruptly between 4 and 8 hrs. returning to the level of the control group after 12 hrs.

The most useful design on which to base the assay is probably that described above, in which a second change of water is made after the mechanical effects of the injection are complete and in which the response to TSH is more clearly defined than under the alternative conditions discussed. It has been shown in these preliminary investigations that an increase in the rate of discharge of ^{131}I can be obtained in response to a single injection of TSH at a concentration of 10 imu/ml., i.e. an absolute dose of 0.2 imu. This figure compares favourably with that obtained for the senstivity of the method of estimation of TSH based on ^{131}I-uptake by the tadpole thyroid. Under the conditions employed in the latter technique, five daily injections of 0.01 ml. were given; the total amount of TSH administered was therefore 0.5 imu.

The advantages of this type of estimation are mainly those of technical simplicity and rapidity. No increase in the order of response obtained with a given concentration of TSH was evident when multiple injections were administered. The necessity for an injection period of several days, therefore, does not arise. Mortality among the test-animals is also very much lower with a single injection than with multiple injections. The response-time (24 hrs. after injection) is comparatively short and the number of samples on which measurement of the radioactivity must be made is small. Because a single measurement is made on the test-group as a whole the problem of individual variation in the results is not encountered.

However, the method is also subject to a number of limitations. The large amount of iodine retained in the extrathyroidal tissues diffuses into

the water and tends to mask the response to TSH. It might reasonably be expected that a specific release of ^{131}I in response to injected TSH would be mirrored in a reduced amount of iodine remaining in the glands at the end of the experiment. It was not possible to demonstrate a significant reduction in thyroid radioactivity in the experimental group as compared with the control group. This may be attributed to individual variability in iodine uptake as a result of which any slight change in thyroidal iodine uptake levels would not be demonstrable. Furthermore, the expected response to a single injection of 0.02 ml. of TSH at a concentration of 10 imu/ml. would be of a low order of magnitude in view of the findings in the preceeding investigations of iodine uptake in response to multiple injections of TSH at this strength. Yet another disadvantage of this particular assay procedure lies in the fact that, should any animals in a group die during the course of the experiment, that entire group must be discarded. The presence of a dead animal in the water on which iodine discharge is measured results in an erroneous estimation of the rate of discharge when iodine is released as the body decomposes.

In conclusion, it may be said that the method of estimation of TSH by ^{131}I-discharge is suitable for application where the concentration of TSH to be assayed is of the order 10 imu/ml. or more. All the possibilities of this type of estimation have not been fully examined. It is proposed that future work should be concerned with investigation of the use of carrier iodine as a means of preventing recirculation of discharged ^{131}I. By this means improved sensitivity and discrimination should be obtained.

S U M M A R Y

SUMMARY

The work described in the preceding chapters was undertaken to determine whether the tadpole of Xenopus laevis is a suitable test-animal for measuring TSH in biological fluids. It was intended that the assay should be applicable on a routine basis for use in investigation of thyroid disorders and the findings were assessed accordingly.

Studies using a histometric technique demonstrated that this method reaches the required standards of sensitivity and reliability when large test-groups are employed; accordingly, it has been used to estimate the concentration of TSH in serum. The limiting factor, where clinical application of the method is concerned, was found to be the time and labour involved in completing the estimation. An attempt was made to overcome this difficulty by application of a projection technique whereby the time required to make cell-height measurements was reduced and the method made more objective. Although, from the point of view of technical simplicity, the histometric method does not compare with either of the radiometric techniques described, it has a lower limit of sensitivity of 0.1 imu/ml. of TSH and has yielded meaningful results in assays on serum. It may be concluded, therefore, that the histometric method can be employed in this type of work provided that the time required to complete an estimation is not of major importance.

The factors influencing thyroidal iodine accumulation were investigated with the aim of developing an assay method based on estimation of ^{131}I-uptake by the glands. A considerable individual variability in iodine uptake was demonstrated in both Xenopus larvae, reared under artificial conditions, and in larvae of the common toad, collected from their natural surroundings. Although neither diet nor temperature was

found to influence this variability greatly, pretreatment with a dilute solution of potassium iodide was found to be effective to a limited extent. In animals pretreated with potassium iodide it was possible to demonstrate an increase in ^{131}I-uptake in response to administration of TSH at a concentration of 10 imu/ml.

Administration of TSH was also shown to have an effect on discharge of ^{131}I as demonstrated by increase in the radioactivity released into the water in which the animals are immersed. This method of measuring thyroid stimulation is indirect and probably less accurate than direct determination of the activity present in the glands. It is further complicated by the presence of comparatively large amounts of iodine retained in the extrathyroidal tissues.

Improvement in the senstivity of the radiometric techniques is entirely dependent on control of individual variability in iodine uptake. In their present form, however, both the ^{131}I-uptake method and the ^{131}I-discharge method are suitable for application where the concentration of TSH is 10 imu/ml. or more, and where very little material is available. Such a situation arises in comparative work on vertebrate endocrinology where small quantities of pituitary material are available in which the expected concentration of TSH would be high as compared with that in serum samples.

PART I. Survey of the Literature

1. A large number of methods for estimation of TSH have been proposed. The reliability criteria of some of these methods are reviewed and their applicability to clinical studies discussed.

2. The chief difficulty in the clinical application of TSH assays lies in the lack of methods of sufficient sensitivity to detect the hormone in the very small quantities in which it is present in body fluids.

3. Results of TSH assays should now be expressed in terms of the International Standard. Much of the confusion which exists in the literature arises from the use of various "animal units" and from the fact that results obtained in one centre cannot be compared with thos in another.

4. Techniques depending on radiometric criteria are generally sensitive, rapid and simple to perform. For these reasons they are probably the procedures of choice for clinical investigations.

5. In euthyroid subjects TSH levels in blood are low and range from zero to 1.5 imu/ml. of serum. In patients with untreated myxoedema higher levels are generally found; these lie in the range 2.0 to 8.0 imu/ml. In thyrotoxicosis, the TSH concentration in serum is variable but frequently found to be higher than that in euthyroid subjects; values quoted in the literature range from zero to 0.5 imu/ml. No correlation has been demonstrated in patients with exophthalmos, between the degree of exophthalmos and the level of circulating TSH.

6. Future work in the field of TSH investigations will probably be concerned with the development of further <u>in vitro</u> assay methods and with the improvement of fractionation techniques by means of which concentrated extracts containing the hormone can be prepared from blood and urine.

PART II. Histometric Methods

1. A histometric technique for assay of TSH using the larva of *Xenopus laevis* was found to have a lower limit of sensitivity of 0.1 imu/ml. The index of precision was reasonably satisfactory when test-groups of ten or more animals were used, the figure for λ being 0.38.

2. The method was used in estimations of the TSH concentration in serum from subjects in whom the TSH-titre was expected to be higher than in euthyroid subjects. The results obtained were found to be comparable with those quoted by D'Angelo *et al.* (1950) and Di George *et al* (1957).

3. It is concluded that, although the technique is time-consuming and laborious, it is suitable for estimation of TSH in body fluids, under conditions where the time required to complete an estimation is not of major importance.

4. An attempt was made to obtain greater rapidity and objectivity by employing a projection method. The results obtained were not entirely conclusive. Further investigation of this technique should result in the development of a method more suitable for routine application in clinical studies.

PART III. Factors influencing ^{131}I-uptake

1. The pattern of ^{131}I-uptake in the untreated tadpole during metamorphosis consisted of a gradual increase in the amount of ^{131}I accumulated in the glands up to the time of eruption of the forelimbs, followed by a decline to the original low level in the newly metamorphosed toad. This was interpreted as indicative of a period of synthesis and storage of hormone, continuing up to the time of onset of shrinkage and change of shape, followed by discharge of the stored hormone during the latter stages of metamorphosis. A similar pattern of uptake was observed in larvae of Bufo and of Rana temporaria.

2. Individual variability in ^{131}I-uptake between larvae of similar hind-limb lengths was considerable in both the Xenopus larvae reared under artifical conditions and in the Bufo larvae obtained from their natural surroundings. Close correlation was found to exist between increase in ^{131}I uptake and increase in hind-limb length in both groups. The variability encountered can not, therefore, be attributed to the artificial methods employed to obtain Xenopus larvae.

3. A trend in the direction of increase in ^{131}I-uptake in relation to increase in both body weight and thyroid size was observed.

4. An attempt was made to determine the amount of ^{131}I accumulated per unit wet weight of thyroid tissue. It was not possible to obtain an exact figure for individual thyroid weights using a conventional balance, but it appeared that the ability of the thyroid to concentrate iodine, as compared with that of extrathyroidal tissue, was ten times greater in animals at the peak of metamorphic activity than in animals at early hind-limb stages.

5. The high degree of variability between animals at the same stage of development, encountered throughout these investigations, was found to be only slightly influenced by diet and temperature. To obtain maximum reduction in variability animals were reared on a mixture of liver powder and dried yeast

and experiments were conducted at 15°C. Extremes of temperature were found to cause pituitary failure and, in consequence, a reduction in ^{131}I-uptake by the thyroids.

6. Starvation was also found to cause a slight reduction in variability. In the "stasis" animal, the amount of iodine taken up was lower than in fed animals as a result of thyroid atrophy.

7. It was concluded that, for assay work, animals with hind-limb lengths <8 mm. should be most suitable for estimations dependent on ^{131}I-uptake and larger animals, with hind-limb lengths >8 mm., should be used in estimations dependent on ^{131}I discharge.

PART IV. Assay by ^{131}I-uptake

1. A measurable response of increase in uptake of ^{131}I by animals immersed in a dilute solution of radioactive iodine was elicited by injection of TSH at a concentration of 10 imu/ml. However, individual tadpoles are so variable in this response, that the discrimination between doses is rather poor. In its present form, therefore, the method is not sufficiently sensitive to be of use in investigation of serum TSH levels, although it could be applied in estimation of TSH in more concentrated extracts.

2. Pretreatment of the experimental animals with a solution of iodine in potassium iodide, potassium iodide alone and thyroxine, all resulted in an enhanced response to TSH stimulation. Potassium iodide in solution was selected as the most effective form of pretreatment since it has no apparent effect on metamorphosis and causes a less drastic reduction in the rate of uptake of iodine by the glands than results after pretreatment with a solution of iodine in potassium iodide.

3. Immersion of the test-animals in ^{131}I throughout the injection period was found to be more effective than immersion at the end of the injection period.

4. It was found that a schedule of twice daily injections was not suitable for routine use since the extra handling involved gives rise to an increased rate of mortality in the test-animals. Increasing the number of injections administered in a given time was not found to result in an augmentation of the response to TSH.

5. Results obtained in investigation of the response to TSH stimulation after injection of ^{131}I were inconclusive. Further study of this method of administering ^{131}I is indicated before it can be dismissed as inferior to the method of immersion.

6. Future work in this field should be concerned with a more detailed examination of the optimum conditions for pretreatment with potassium iodide and investigation of other methods of controlling variability.

PART V. Assay by ^{131}I-discharge

1. In untreated tadpoles transferred to clean water after immersion in a solution of ^{131}I, discharge of ^{131}I occurred until a state of equilibrium was reached between iodine retained in the body and iodine released into the water, 72 hrs. after the change was made.

2. When the water was changed at 24 hr. intervals, a greater amount of ^{131}I was released than when only one change was made. This process continued to occur for at least 120 hrs. after the first change from the ^{131}I-solution. The iodine released under these conditions was considered to be mainly extra-thyroidal in origin.

3. An increase in the amount of iodine discharged was obtained in response to a single injection of TSH at a concentration of 10 imu/ml. It was found that neither the magnitude of the response nor the response time were affected by administering multiple injections.

4. A response-time of 24 hrs. after the injection of TSH was employed. It was found that, whereas with high doses of TSH the maximum response occurred at 6 hrs., with lower concentrations the maximum response occurred between 24 and 48 hrs.

5. In both the experimental group and the control group an increase in discharge of ^{131}I was found to occur immediately after injection as a result of mechanical disturbances due to handling and injection. A more marked response to TSH was obtained when the water was changed for a second time after this mechanical response was complete.

6. In its present form this assay method may be regarded as suitable for use with test-substance in which the concentration of TSH is 10 imu/ml. or more. Future work should be concerned with investigation of the use of carrier iodide as a means of blocking recirculation of discharged ^{131}I and other methods of improving the sensitivity of the method.

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Measurement of thyrotrophic hormone (TSH) using the *Xenopus* tadpole and ^{131}I.

By Judith R. Brown and J. M. Dodd. *Clinical Endocrinology Research Unit, Medical Research Council, University of Edinburgh and Gatty Marine Laboratory, University of St Andrews*

The choice of anuran tadpoles for the investigation of thyroid phenomena is a rational one since the thyroid gland is such an active organ in these animals and since many of the tissues are highly sensitive to thyroid hormone and therefore, indirectly, to TSH. Under the influence of thyroid hormone the hind-legs grow, the body and tail shrink in length and weight, the front limbs emerge and the gut shortens. All these changes are easily measurable and can be used as criteria of TSH-thyroid activity. Further, the histological appearance and metabolic activity of the thyroid glands have been shown to be controlled by TSH. The tadpole has the additional advantage as a test animal that only a small amount of the substance under investigation is required: ten tadpoles can each be injected five times for a total expenditure of only 1 ml. of solution.

The stasis tadpole method for assaying TSH [D'Angelo, Gordon & Charipper, 1942], is well known and is highly sensitive. We have developed a modification of this method using the larvae of *Xenopus laevis* and this offers a similar degree of sensitivity [see also Asboe-Hansen, Iverson & Wichmann, 1952]. However, these histometric methods are tedious and time-consuming, and in an attempt to develop a more simple assay method we have studied the metabolism of ^{131}I by the thyroid glands of *Xenopus* tadpoles under a variety of conditions [Dodd, 1956], and under the influence of thyroid-stimulating hormone. Several difficulties have been encountered, especially a variability between individual tadpoles with respect to the affinity of their thyroid glands for ^{131}I. This variability reduces the degree to which the test can differentiate between different doses of administered TSH.

The method involves the use of tadpoles of *Xenopus laevis*, and these can be obtained in large numbers throughout the year. Tadpoles weighing approximately 500 mg and having hind-legs between 4 and 6 mm in length are selected and starved for 8 days. Starvation has been found by D'Angelo, Gordon & Charipper [1941] to induce a condition of pseudo-hypophysectomy in the tadpoles of *Rana pipiens* which suppresses thyrotrophic function in the pituitaries of the test animals. After starvation and throughout the injection period, groups of ten tadpoles are placed in Kilner jars containing 1 l. distilled water to which 10μc. ^{131}I has been added. The tadpoles are injected once a day for 5 days. At each injection 0·02 ml. of the substance under test in 0·75 % saline is injected into the submental lymph sac, using the interhyoideus muscle as a seal. The control animals receive similar injections of 0·75 % saline. At the end of the injection period each group of tadpoles is placed in clean water and anaesthetized in 1 % urethane immediately prior to thyroid removal. The anaesthetized tadpole is placed on its back on wet cotton-wool on the stage of a low-power binocular microscope. A finely pointed glass rod which concentrates light from a variable intensity microscope lamp is placed in the tadpole's mouth and the thyroid glands located in the bright spot of light which it produces. The thyroid glands are

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then removed by the use of tungsten needles and Dumont forceps, placed on a planchette and counted in standard end-window counting equipment.

The limit of detection of the method as it stands is approximately 10 international milli-units (i.m.u.) TSH/ml., i.e. an absolute amount of 1 i.m.u. A reliable figure is not yet available for the degree of discrimination between doses which the method will give, since there still remains the problem of further reducing variability between tadpoles so that better discrimination becomes possible. This aspect of the work is receiving attention at the moment and a successful outcome, combined with a suitable extraction technique for TSH, would make the method applicable to the study of thyroid-pituitary relationships in man.

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THE BIOSYNTHESIS OF THYROXINE AND 3:5:3′-TRIIODOTHYRONINE IN LARVAL AND ADULT TOADS

By CLAIRE J. SHELLABARGER* and JUDITH R. BROWN

From the National Institute for Medical Research, London, N.W. 7, and Clinical Endocrinology Research Unit (Medical Research Council), Edinburgh

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SUMMARY

1. A study has been made of the compounds synthesized by and present in the thyroid gland of larval and adult *Xenopus laevis* using radioactive iodine and chromatography.

2. Tadpoles undergoing active metamorphosis produced thyroxine and trace amounts of 3:5:3′-triiodothyronine. Less thyroxine and no triiodothyronine was detected in tadpoles approaching the end of metamorphosis, and neither compound was detected in tadpoles in early stages of metamorphosis. Adult toads produced thyroxine and trace amounts of triiodothyronine. The presence of monoiodotyrosine and diiodotyrosine was a constant finding.

3. It is suggested that the synthesis of thyroid hormones in amphibia is similar to that in other vertebrates.

The classical experiments of Gudernatsch [1912], Allen [1916] and Hoskins & Hoskins [1917] have established the role of the thyroid gland in amphibian metamorphosis. Equally, the fundamental discoveries of Kendall [1915], Harington [1926] and Harington & Barger [1927] established the structure of the thyroid hormone—thyroxine—with its complement of 4 atoms of iodine. However, little is known of the nature of the biosynthesis of the thyroid hormone, or hormones, in amphibia, beyond the fact that the thyroid gland of this class of vertebrates is capable of concentrating and organically binding iodine [Gorbman & Evans, 1941; Matthews, 1950]. Although studies of the capacity of the amphibian thyroid gland to concentrate and release radioactive iodine have been reported [Dent, 1952; Dodd, 1955; Money, Lucas & Rawson, 1955; Brown & Dodd, 1956], and although attempts have been made to correlate parameters of thyroid function with the stage of metamorphosis and with thyroid and pituitary cytology [Saxèn, Saxèn, Toivonen & Salimaki, 1957*a*, *b*], little is known about the actual compounds synthesized by the larval or adult amphibian gland. It is the purpose of this communication to present data, obtained by utilizing ^{131}I and chromatography, regarding the qualitative and quantitative analysis of the compounds synthesized by the thyroid gland of the metamorphosing tadpole and young adult toad.

METHODS

Larvae of *Xenopus laevis* were selected for these studies. The thyroid gland can be removed from the tadpole, thus obviating the possible complication of the extrathyroidal synthesis of organic iodine compounds [Hunt & Bailey, 1957]; a supply of larvae in all stages of metamorphic development can be maintained in the laboratory

* Special Fellow of U.S. Public Health Service, NIAMD. Present address: Brookhaven National Laboratory, Upton, Long Island, New York.

throughout the year. Fertilized eggs were obtained by artificially induced ovulation and hatched in running water at a constant temperature of 20–22° C. The larvae were then transferred to a shallow aquarium and fed on a suspension of dried liver powder. When large enough to withstand individual handling, they were transferred to jars containing ten animals in 700 ml. of water in which they were maintained under constant conditions of temperature and illumination and were fed 100 mg of a 50/50 mixture of liver powder and dried yeast every 3 days.

For the present study thirty-eight larvae and five adult toads were selected. Using hind-limb length as a measure of advance in metamorphosis, tadpoles were selected to represent the three major phases in development, ranging from early hind-limb development through the peak of metamorphic activity, at which stage the fore-limbs erupt, to the completion of metamorphosis when the tail is fully reabsorbed. These phases may be described as the 'pre-critical' period of growth of the hind-limbs, the 'critical' period of rapid change recognized by D'Angelo, Gordon & Charipper [1941] and the final 'post-critical' period. The toads used were young adults measuring 2–3 cm from snout to vent.

The tadpoles were exposed to 200 μc. ^{131}I in 1000 ml. tap water for 48 hr at 22° C in groups of three to five animals; all animals in each group were at the same stage of metamorphosis. Adult toads were given either 20 or 100 μc. ^{131}I in 0·03 ml. saline injected into the dorsal lymph sac. They were maintained at 22° C and killed at 24, 48 or 72 hr later. The thyroid glands of the adult toads were studied individually, and since no major difference in results was noted in relation to the amount of isotope administered or to the time after injection at which the animals were killed, the results are expressed as average values based on the five toads.

The thyroid glands of the tadpoles were obtained from animals previously exposed to ^{131}I and pooled in groups of three to five.

After immersion in the radioactive iodine solution for 48 hr, each group of larvae was washed for 30 min in tap water and anaesthetized in a 1 % urethane solution immediately before thyroidectomy. The tadpole was then placed on its dorsal surface on damp cotton-wool on the stage of a binocular dissecting microscope. A pointed glass rod concentrating light from a shielded lamp was placed in the mouth of the tadpole to illuminate from below the region in which the thyroid glands lie. Located in the bright spot of light thus produced, the thyroid glands were removed by use of tungsten needles and Dumont forceps.

The thyroid glands from each group of tadpoles and individual adult toads were placed in tubes containing 0·2 ml. boric acid buffer (pH 8·6) and a small amount of trypsin. The ampoules were placed on a shaker in a room at 35° C for approx. 24 hr. The hydrolysate was applied, in duplicate, to 1 in. strips of Whatman No. 1 filter-paper for ascending chromatography in *n*-butanol-dioxane (80:20, v/v) saturated with 2N aqueous ammonia, together with thyroxine, triiodothyronine, diiodotyrosine, monoiodotyrosine and iodide carriers. These strips were counted throughout their length in an automatic recording beta counter. The carriers were localized with diazotized sulphanilic acid and palladium chloride. The relative percentages of radioactivity corresponding to the positions of the carriers were determined by correlation with the peaks on the tracing obtained in the counter. In addition, the hydrolysate was applied to 6 in. strips of paper for ascending chromatography

in *n*-butanol-acetic acid-water (78:10:12, v/v), with the appropriate carriers. The positions of the carriers were visualized in U.V. light, the triiodothyronine area of the paper was cut out, eluted with methanol-ammonia and rechromatographed in two dimensions in both systems, with thyroxine and triiodothyronine carriers. These chromatograms were radiographed, the positions of the carriers were localized as above, and the presence of radioactivity was correlated with the positions of the carriers.

RESULTS

It can be seen (Table 1) that significant amounts of thyroxine are produced in the glands of the young adult toad. In tadpoles in the 'critical' period of rapid metamorphosis, somewhat smaller amounts of thyroxine were found. The amounts were

Table 1. *Amount of thyroid hormone present in glands of the tadpole of* Xenopus laevis *and in the young adult toad*

No. of animals in group	Stage in development	Average hind-limb length (mm)	Percentage distribution of ^{131}I on chromatogram			
			Thyroxine area	MIT and DIT*	3:5:3′-Triiodothyronine	I
5	'Pre-critical'	2·3	—	4	—	96
4		2·7	—	3	—	97
4		4·0	1	36	—	63
4	'Critical'	5·3	5	28	Trace	67
4		6·2	1	49	—	49
3		8·6	7	30	Trace	63
4		9·7	6	30	Trace	64
10	'Post-critical'	21·0	3	77	—	20
5	Young adult toad	—	10	79	Trace	8

* MIT = monoiodotyrosine; DIT = diiodotyrosine.

still smaller in animals in the 'post-critical' phase, while in animals in the 'pre-critical' phase the presence of thyroxine could not be detected. 3:5:3′-Triiodothyronine was identified in trace amounts only in thyroid glands from adult toads and from tadpoles showing relatively large amounts of thyroxine. Monoiodotyrosine and diiodotyrosine could be demonstrated both in glands from those animals which did and those which did not show thyroxine.

DISCUSSION

A number of studies have established that metamorphosis depends on a normal pituitary-thyroid relationship [Adler, 1914; Allen, 1916; Smith, 1920] and that thyroid hormone or thyroid hormone-like substances induce metamorphosis [Gudernatsch, 1912]. These studies, and many others, have shown that metamorphosis is dependent on the production of thyroid hormone, although there has been no direct demonstration that the thyroid gland of the intact, untreated tadpole produces thyroid hormone or hormones during metamorphosis.

The chromatographic data provided by the present study demonstrate that the thyroid gland of the *Xenopus laevis* tadpole does produce thyroxine and trace

amounts of triiodothyronine. Furthermore, it is apparent that thyroid hormone synthesis is greatest at the time when the tadpole is undergoing the most rapid metamorphosis, as indicated by the increased growth rate of the hind limbs and other morphological changes. It is of interest to note that Saxèn *et al.* [1957*a*, *b*] have suggested that the most rapid uptake and release of thyroidal radio-iodine during metamorphosis of this species of tadpole occur at approximately the same stage of metamorphosis at which, in the present study, largest amounts of thyroxine were demonstrated.

The finding that glands from larval and adult toads produce monoiodotyrosine and diiodotyrosine would suggest that the synthesis of thyroid hormones in amphibia is similar to that in other classes of vertebrates.

Although it is reasonable to assume that thyroid hormones play a part in the metamorphosis of amphibia, the physiological activity of thyroid hormone in the adult remains obscure [Lynn & Wachowski, 1951].

Since with this report 3:5:3′-triiodothyronine has now been found in all classes of vertebrates, further support is added to the suggestion that triiodothyronine should be considered as one of the thyroid hormones.

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